The Journal of Laboratory and Clinical Medicine

VOL. 27 JANUARY, 1942 NO. 4

CLINICAL AND EXPERIMENTAL

SYSTEMIC HISTOPLASMOSIS*

SYSTEMIC HISTOPLASMOSIS DIAGNOSED BEFORE DEATH AND PRODUCED EXPERIMENTALLY IN GUINEA PIGS

J. D. REID, SC.D., J. H. SCHERER, M.D., P. A. HERBUT, M.D., AND H. IRVING, M.T.
RICHMOND, VA.

THE discovery by Darling[1] in 1906 and the subsequent report of three cases of histoplasmosis in Panama has been followed by the observation of seven cases of this fatal systemic disease in this country. We are recording here the eighth case of systemic histoplasmosis that has occurred in the United States.† Diagnosis was made from the blood smear and blood culture during life. The disease in a systemic form also has been transmitted to guinea pigs for the first time.

Between the years 1906 and 1909 Darling[2] reported three cases of a systemic parasitic infection, the causative organism of which he named *Histoplasma capsulatum*. In each case diagnosis was made following autopsy, and Darling, because of the clinical picture and the resemblance of the parasites to Leishman-Donovan bodies, believed it to be a tropical disease related to kala azar. Not until 1926 was the next case recorded, and this by Riley and Watson,[3, 4] was the first to be reported from North America. In the same year, Phelps and Mallory[5] reported a case of "histoplasmosis of the lung," but neither the disease nor the pathology of this case resembled that of systemic histoplasmosis. A fifth case was found in an American negro in 1931 and was reported by Crumrine and Kessel.[6] In all these cases the diagnosis was made only after autopsy, and attempts to culture the parasite were unsuccessful. Dodd and Tompkins[7] dis-

*From the Departments of Bacteriology, Medicine, and Pathology, Medical College of Virginia.

†Since this report was written seven additional cases of systemic histoplasmosis have been published. Meleny, H. E. (Am. J. Trop. Med. 20: 603, 1940), reviewing the literature, records thirteen unpublished cases, four of which appear to be systemic.

Received for publication, June 18, 1940.

covered in 1934 the sixth case in an infant from Tennessee. The diagnosis was made in this case for the first time during life by the finding of the parasites in the monocytes of the blood. For the first time, likewise, the organism was successfully cultivated from material obtained at autopsy, and DeMonbreun[8] was able to produce the disease in monkeys by intravenous injection of cultures.

Subsequently, Agress and Gray,[9] Amolsch and Wax,[10] Shaffer, Shaul, and Mitchell,[11] and Clemens and Barnes,[12] reported cases of systemic histoplasmosis in which diagnosis was established at postmortem by the finding of the parasites within the cells of infected organs. Culture of the parasite from the spleen was obtained in only one of these four cases, that reported by Clemens and Barnes.[12]

Clinically, systemic histoplasmosis, as described by Darling, is characterized by "splenomegaly, emaciation, irregular pyrexia, leukopenia, and anemia." Only in the case of Crumrine and Kessel was splenomegaly absent. The pathology has been described as "the invasion of endothelial cells in the smaller lymph and blood vessels and capillaries by enormous numbers of a small encapsulated organism (*Histoplasma capsulatum*), causing necrosis of the liver with cirrhosis, splenomegaly, pseudogranuloma of the lungs, small and large intestines, with ulceration of the latter, and necrosis of the lymph nodes draining the injected viscera."[13]

CASE REPORT

The patient, a 38-year-old negro man, was referred to St. Philip Hospital, on March 23, 1939, for diagnosis. His chief complaint was "weakness," a symptom which had become progressively more pronounced since it was first noticed in mid-December. A janitor in a real estate office, he had continued at work until the day before admission, in spite of an increasing weakness of his arms and legs, which steadily increased the effort required in the performance of his routine duties. He had had a loose cough with slight expectoration since January, and had noted profuse sweating, associated at times with the sensation of "chills." The sweats were noticeable especially at night, often being of a severity sufficient to make him get up and change his clothing. For two months previous to his admission to the hospital he had been unable to walk more than a city block without sitting down to rest because of dyspnea and weakness in the knees. His appetite had grown progressively poorer, and during the last week he had drunk only milk. Hoarseness also had been noted during the last week. The patient believed he had lost about 50 pounds in weight, and for three weeks had been troubled with a watery diarrhea.

Past history was nonessential, except for the observation that the patient, born in South Carolina, had lived in Richmond, Va., for the past twenty years and had had no known contacts with the tropics. His health had been good until the present.

Physical Examination: The patient's skin was hot and moist. His mucous membranes were pale. He was very cooperative but a marked hoarseness made difficult his response to questioning. His temperature was 102° F., pulse rate 108, and respiration, 22 per minute. Examination of the eyes revealed small, white, irregular areas surrounded by hemorrhages in both fundi. These were not unlike tubercles. The pharynx was reddened. On laryngeal examination there was seen to be edema of both arytenoids with hyperemic cords which did not approximate well. The right cord especially was roughened, but there were no ulcerations. Lymph glands in the axillary, cervical, submental, and popliteal areas were enlarged, soft, discreet, and nontender. Chest examination revealed moist râles in both bases posteriorly and bronchial breathing on the right, anteriorly, just below the nipple area. With the exception of a soft systolic murmur, which was heard over the apex and not transmitted, heart examination was negative. The blood pressure was systolic 124 and diastolic 60. The abdomen was negative; neither the spleen nor the liver was palpated. There were no alterations in reflexes. The admission impressions were miliary tuberculosis, bacterial endocarditis, and Hodgkin's disease.

Laboratory Examination: Urinalysis: specific gravity 1.013, trace of albumin, occasional pus cell and hyaline cast. Blood: red cells 2,540,000; hemoglobin 54 per cent; white cells 3,200; differential count: neutrophiles, 68 per cent, lymphocytes, 28 per cent, monocytes 4 per cent. Bleeding and clotting times were normal. Blood chemistry: sugar 141 mg., nonprotein nitrogen 25 mg., calcium 7.8 mg., phosphorus 3.1 mg., total protein 4.7 mg., albumin 2.1, and globulin 2.6. Complement fixation and flocculation tests were negative. Spinal fluid, stool, and agglutination examinations were also negative.

Roentgenologic examination of the chest showed "irregular mottling on the right first and second interspaces anteriorly with moderate density of the hilum and trunk shadows on the right, suggesting definite, minimal tuberculosis of the right lung."

In the hospital the patient ran a septic type of temperature which was characterized by an irregular regularity. This ranged between 100 and 103 degrees, rising to the higher level between 4 and 12 P.M. and falling during the night and early morning, only to rise again in the late afternoon or evening. This occurred daily without exception until the final week of his illness, when the temperature fluctuated between 99 and 101 degrees with the higher level still in the afternoon.

The patient continued to grow weaker, and, when repeated sputum examinations were negative for the tubercle bacillus, the hematologic service was consulted because of the anemia.

Study of the blood smear showed no malarial parasites. However, because of the presence of an endothelial type of monocyte, one of us (H. I.) continued to examine the smear further, the search being finally rewarded by the finding of a large phagocytic cell containing numerous small bluish hyaline bodies which, at the time, were not identified. The report on this examination was: "Blood smear shows slightly regenerative, hypochromic, microcytic anemia with rare normoblasts. There is a left shift at the stab level and the neutrophiles show slight toxic granulation with frequent Döhle bodies. An occasional 'blast' cell is seen and the platelets appear greatly reduced. On checking, these were found to number 122,000 (normal 500,000). Phagocytic monocytes are present and rarely one of these is seen to contain numerous small bluish hyaline bodies with dark staining nuclei. These measure 2μ to 4μ and vaguely resemble Leishman-Donovan bodies."

Sternal puncture was resorted to for further study of the blood. The bone marrow aspiration showed numerous monocytes filled with the parasites, and there were numbers of neutrophiles containing from one to five organisms. The marrow was moderately erythroblastic and contained a number of plasma cells. Megakaryocytes were notably decreased. In addition to the parasites there were phagocytosed red and white blood cells in the large endothelial cells, many of which were binucleated.

Following this finding, an axillary lymph node was removed. This showed a chronic lymphadenitis with proliferation of the reticulum, the reticulum cells, under oil immersion, being engorged with numerous inclusion bodies which possessed a pale peripheral halo and a faint oval basophilic center.

A previously negative blood culture was re-examined and a fresh culture was taken. These were examined for fungi and found to be positive for *Histoplasma capsulatum*. In the meanwhile daily blood smears continued to show the parasites in increasing numbers. While at first the parasites had been observed only in the monocytes, as the disease progressed the neutrophiles were found to contain them. There was a steady increase in toxic degenerative granulation of the leucocytes, as normoblasts and myelocytes began to appear in greater numbers, and the patient, becoming gradually weaker, died in stupor on April 24, 1939.

POST-MORTEM EXAMINATION

(Abstract from Protocol)

Externally the only finding of interest was that of a moderate swelling of lymph nodes in the axilla, groin, and neck. No skin lesions of any type were noted.

Thorax: The right pleural cavity contained approximately 500 c.c. of clear straw-colored fluid. There was no effusion into the left pleural cavity or the pericardial cavity.

Lungs: The base of the right upper lobe in its lateral aspect showed a depressed stellate grayish-white area, measuring approximately 0.5 cm. in diameter in its central portion. Sections through this lesion showed a roughly triangular area slightly raised, measur-

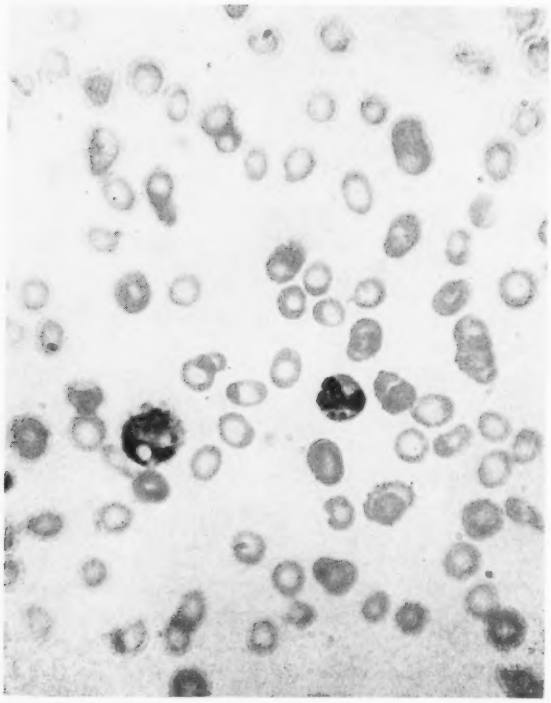


Fig. 1.—Peripheral blood showing parasites in polymorphonuclear cells.

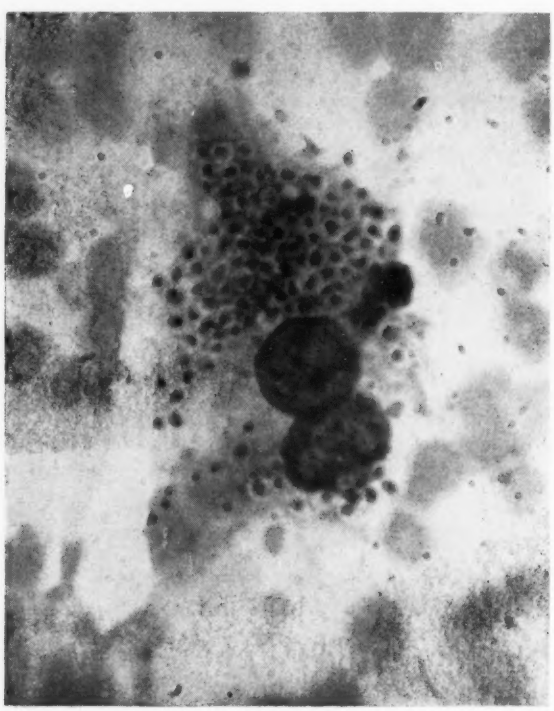


Fig. 2.—Large binucleated endothelial cells containing numerous parasites. Sternal bone marrow.

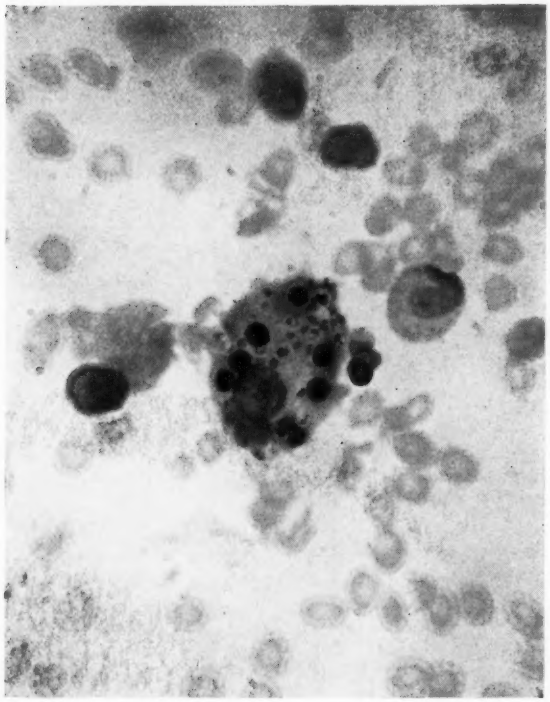


Fig. 3.—Endothelial cell from bone marrow puncture is shown with engulfed normoblasts and parasites.

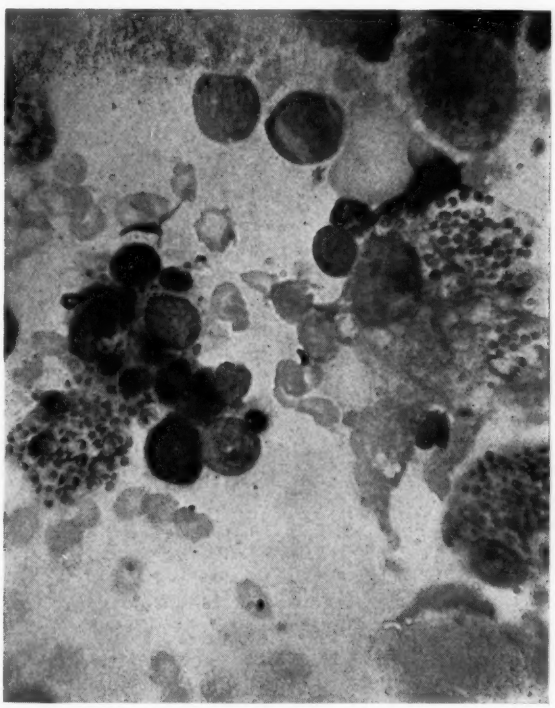


Fig. 4.—Numerous endothelial cells with parasites seen in parts of smear from sternum.

ing about 2 cm. at its pleural base and 3 cm. from the base to its apex near the hilus. This area of infiltration was composed of discrete and frequently conglomerate nodules, measuring from the size of a millet seed to 3 mm. in diameter. They were situated for the most part peribronchially, but did not show rosette formation or any other distinct pattern of distribution. On cut surface the nodules were raised, grayish white in color and partially caseous in their center. The margins of the larger confluent areas of grayish-yellow necroses were firmer and presented a somewhat gritty sensation when scraped with a knife. The intervening tissue was slightly depressed beneath the cut surface with grayish-white prominent bands. There was abundant anthracosis in this area. No abscesses were seen. The remaining parenchyma of both lungs was normal. The hilar lymph nodes were moderately enlarged and contained carbon pigment; they failed to disclose distinctly visible nodules or areas of caseation.

Spleen: The spleen weighed 500 Gm. and measured 14 by 11 by 7 cm. It was firmer in consistency than normal. The pulp did not scrape away on the knife edge. The follicular and trabecular structures were obscured.

Kidneys: Each kidney weighed 200 Gm. and measured 10 by 7 by 4 cm. Their surfaces were very finely granular. The cut surface was very moist. There was an eversion of the cut edges. The corticomedullary demarcations were markedly obscured. The architecture of the medulla and cortex proper appeared to be normal.

Liver: The liver weighed 2,400 Gm. Its dimensions were 27 by 18 by 10 cm. Grossly it presented no abnormalities.

Intestines: The lower 45 cm. of the ileum, the cecum, and the ascending colon showed scattered mucosal nodules, measuring from 2 to 5 mm. in diameter, raised about 2 mm. above the surface. The smaller nodules were grayish white. On sectioning they were firm and solid. The larger ones showed central areas of caseation. In the cecum and ascending colon were found numerous areas of ulceration, mostly round in shape. Their margins were sharp but ragged and surrounded by a hemorrhagic zone. They did not seem to be undermined. The ulcers measured from 1 mm. to about 5 mm. in diameter.

Lymph Nodes: The mesenteric lymph nodes were moderately enlarged. The paraaortic lymph nodes and lymph nodes of the liver hilus were markedly enlarged, matted together in irregular masses adherent to the adjacent tissues. They were firm to palpation and on section showed irregular well-defined areas of yellowish necrosis.

Vertebral Bone Marrow: Showed grossly no abnormalities.

MICROSCOPIC EXAMINATION

Organisms: Characteristic organisms of *Histoplasma capsulatum* were found in the lungs, spleen, kidney, intestines, liver, pancreas, lymph nodes, and bone marrow. The organism itself was usually round, or slightly oblong, in shape, and measured approximately 2μ to 4μ in diameter. In both the ordinary hematoxylin-eosin stain and in Goodpasture's stain the external membrane was nonstaining and doubly refractile. The internal structure of the organism was variable. It contained a clear vacuolated nonstaining cytoplasm and a bluish-stained chromatin material, the arrangement of which varied greatly. In some of them the chromatin material was finely granular and found in the center of the body; in some it was situated at one or both poles in the form of small and larger round masses; in others these unipolar or bipolar masses were more crescentic in shape and partially encircled either one or both poles. In still others they seemed to encircle the periphery entirely. In all bodies the remaining portion of the organism was composed of a nonstaining cytoplasm which was negative for both Ziehl-Neelsen and fat stains. For the most part the organisms were found ingested in cells, but occasionally they were found free, singly and in groups.

Typical Lesion: The typical lesion produced in the various organs by *Histoplasma capsulatum* was a rather characteristic nodular granuloma resembling a tubercle or a gumma. The nodules varied in size from 15μ to 20μ to several millimeters in diameter. The smaller were circular in outline, the larger appeared to be confluent, disclosing serpiginous borders or a maplike outline. The central portions were composed of eosinophilic staining, partially granular, unidentifiable material. Some foci of necrosis contained many nuclear fragments.

In many of them, this central area of necrosis still revealed a shadowy outline of the underlying structures. Capillaries were found to be coursing through the entire nodule and were often seen to be patent and filled with blood, even in the center of the necrosis. The central necrotic areas frequently disclosed the structure of elastic membranes still completely or partially intact. The alveolar septa and vessels could easily be recognized within the areas of necrosis by elastic tissue stains. At the periphery the necrotic areas shaped themselves into ill-defined faintly staining "ghosts" of markedly engorged mononuclear cells. Still more peripherally the large monocytes were well preserved. They contained ingested material which was composed of large numbers of closely packed *Histoplasma capsulatum* organisms. About the periphery of the nodules were scattered plasma cells, lymphocytes, and a few polymorphonuclear cells. Giant cells and epithelioid cells were absent.

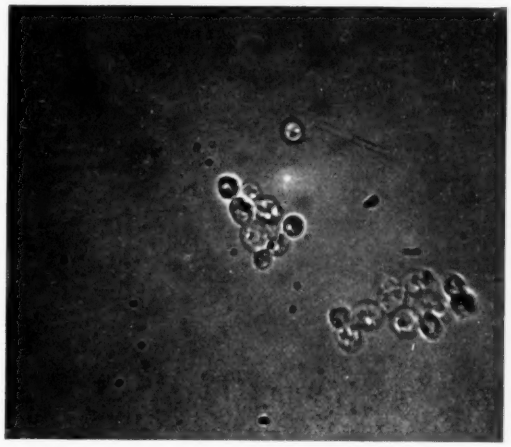


Fig. 5.—Yeastlike forms of *Histoplasma capsulatum* from a six-day-old culture on sealed blood agar slant at 37° C. Wet mount (×700).

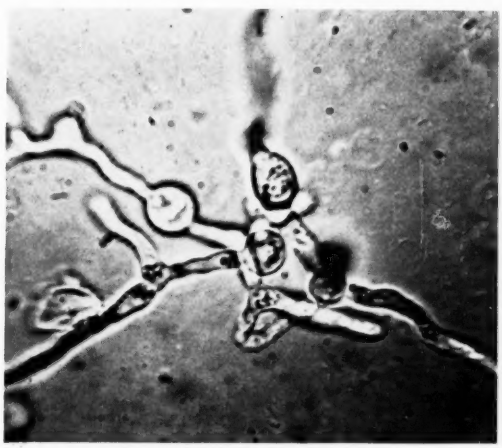


Fig. 6.—Hyphae developing from yeastlike form in semisolid culture media containing fresh blood at 37° C. Wet mount (×700).

Lungs: Sections through the lesions at the base of the upper lobe of the right lung showed many of the typical granulomas described. Here they were mostly confluent. In one area a cytoplasmatic mass was encountered which resembled a Langhans' giant cell: vesicular nuclei were grouped around a pink-staining cytoplasm. It could not, however, be definitely identified as a foreign body giant cell. In addition to the above-described typical lesion there was a marked polymorphonuclear cell infiltration in a diffuse form in the

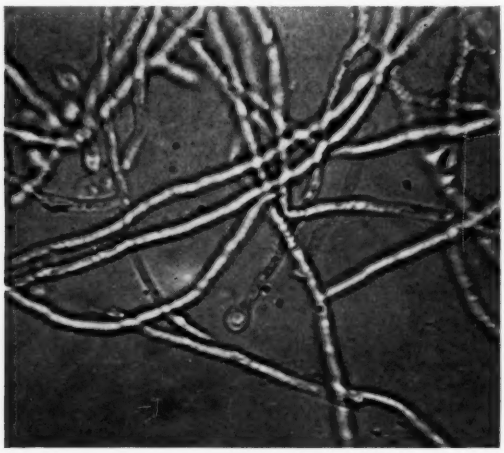


Fig. 7.—Early mycelial type of growth in same media as Fig. 6 at room temperature. Wet mount (×700).

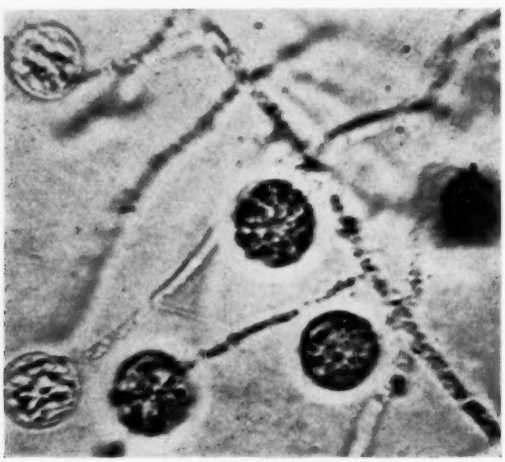


Fig. 8.—Development of mycelial form on dextrose agar plates at room temperature, showing enlarging arthrospores. Wet mount (×700).

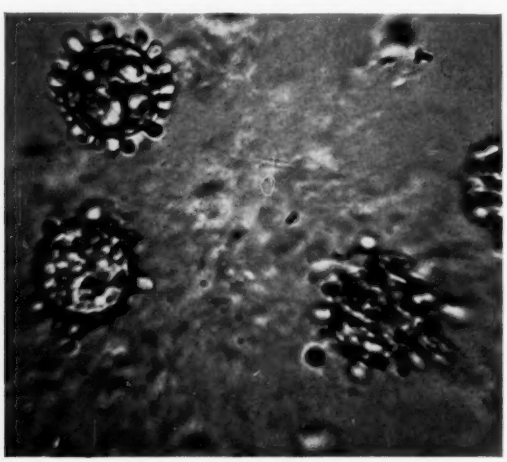


Fig. 9.—Further development of arthrospores showing the tuberclelike protrusions. Wet mount (×700).

interalveolar septa. There were also discreet areas of dense infiltration with polymorphonuclear cells, which showed a liquefactive necrosis of the underlying tissues. These possessed all the characteristics of a true, though small, abscess. Marked interstitial infiltration with lymphocytes and plasma cells was found in the vicinity of the above-described granulomatous nodules in the interalveolar septa. There was extensive fibrous scarring throughout. A number of large discrete areas were encountered which resembled old-healed conglomerate tubercles. In fact, from a histologic point of view, they could not be distinguished from the latter. It is uncertain whether or not such foci should be looked upon as a healing phase of the granulomas caused by *Histoplasma capsulatum*.

Vessels were involved in the inflammatory processes. Arteries of various sizes were seen with proliferating endarteritis and complete obstruction of their lumina. The granulation tissue in many areas involved medium-sized and smaller veins. Elastic tissue stain disclosed that all layers of the wall were infiltrated by a variety of small round cells. The elastic lamellae, in particular those of the adventitial layers, were "teased up," split apart, and partially disintegrated. The process encroached upon the media and resulted in disintegration of the wall and segmental disappearance of elastic fibers. Many veins showed proliferation of subintimal cells with formation of intimal pads. In some of these the granulation tissue completely occluded the lumen.

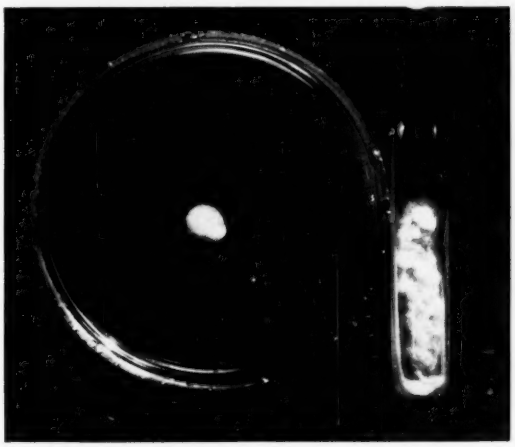


Fig. 10.—Three weeks' old culture on Sabouraud's dextrose agar at room temperature. The aerial hyphae which give the colony its fluffy appearance are plainly visible. Wet mount (×0.5).

Spleen: The spleen showed many minute discrete nodules of the typical type. Polymorphonuclear cell reaction, however, within or in the vicinity of these granulomas was rather scarce. The sinusoids were dilated and contained large monocytes and desquamated reticulum cells, both of which were markedly engorged with organisms. There was diffuse hyperplasia of the reticulum which was best seen in silver stains.

Pancreas: The pancreas showed a slight diffuse lymphocytic and plasma cell infiltration of the somewhat edematous interstitial connective tissue. There were also scattered macrophages that contained the ingested organisms. Characteristic nodules were not encountered.

Kidneys: The kidneys did not show specific nodules. The organisms, however, were occasionally found in large cells in the glomerular tufts. It could not be determined whether these cells were endothelial or epithelial. The interstitial tissue of the cortex showed edema and a moderate infiltration with plasma and round cells. Occasional polymorphonuclear cells were also encountered.

Liver: The liver contained many minute and larger typical *Histoplasma capsulatum* nodules. They bore no special relation to the liver lobules, some being found in the portal areas, others midway between the center and the periphery of the lobules, and still others

occupying most of the lobule. In addition, Kupffer cells throughout the entire liver substance were greatly increased in size and contained the organisms. Otherwise the liver was essentially negative.

Intestines: The ulcers about the ileocecal region were rather superficial, and, as a rule, did not extend deeper than the submucosa. The edges were rather sharp, not undermined. The floor and margins were composed of necrotic tissue with nuclear fragments and "ghosts" of large mononuclear cells with phagocytosed organisms. Beneath the floor was found a layer of nonspecific granulation tissue that contained many mononuclear macrophages with ingested organisms, a variety of larger and smaller round cells, fibroblasts, and capillaries. Polymorphonuclear cells were found abundantly within this granulation tissue. The involvement of the vessels was of the same type as that described in the lungs.

Lymph Nodes: They contained many typical granuomas already described. Reticulum cells throughout were very conspicuous, being greatly engorged with organisms. The follicles were small and widely separated by diffuse hyperplastic reticulum. Polymorphonuclear cells were scarce. Many veins in the periphery were engorged with macrophages that contained the organisms. Periendophlebitis, as described in the lung, was likewise encountered in the vicinity of the caseous granulomas.

Bone Marrow: Rather actively functioning, the bone marrow contained numerous large mononuclear macrophages and reticulum cells engorged with the organisms.

Brain: Permission for examination of the brain was not given, and it was, therefore, not possible to examine the eyes.

The remaining organs of the body were essentially negative.

MYCOLOGIC STUDIES

The clinical symptoms of histoplasmosis are so ill-defined that only one of the ten cases published previously has been diagnosed before the death of the patient. In this case, presented by Dodd and Tompkins,[7] the typical yeastlike bodies of *Histoplasma capsulatum* were found in the smears of the blood. Later, the fungus was also cultivated from the blood by DeMonbreun.[8] Clemens and Barnes[12] have also reported finding the parasite in a blood culture taken on their case and examined one month later after diagnosis was established at autopsy. Since the ante-mortem diagnosis of this infection rests, at the present time, upon these findings, we are presenting the cultural methods used in obtaining the parasite from this case of systemic histoplasmosis.

The blood culture media utilized for routine work in the laboratory and which we used in this study is prepared as follows: Veal infusion broth containing 0.3 per cent dextrose, 0.5 per cent sodium citrate, and 0.2 per cent agar adjusted to pH 7.4 is sterilized in 40 c.c. amounts in small Erlenmeyer flasks. Usually 10 c.c. of blood are taken from the patient and inoculated directly into the flask. Incubation is carried out aerobically or anaerobically, as the case may warrant, at a temperature of 37° C.

The first blood culture was received from the patient four days after admission to the hospital, and approximately fourteen weeks after the onset of symptoms. This culture was found to be contaminated and was discarded at the end of four days' incubation. A second blood culture was received three days later. There were noticed in this flask at the end of twelve days' incubation numerous yeastlike bodies and a few scattered mycelial threads. One of us (H. I.) had found similar bodies somewhat earlier in smears prepared from the peripheral blood. These bodies were always found within the cells, particularly within the cytoplasm of the large monocytes. The yeastlike cells in the blood culture media tended to adhere to one another after division and were

often found in small clusters, particularly in wet preparations. One or two dark granules were visible within the otherwise clear cytoplasm of these unstained cells. When stained by Wright's method, the organisms were almost identical in appearance to those occurring in the tissue cells. They appeared as oval, occasionally round, cells, 2.7μ to 3.6μ in their longest diameter, exclusive of the capsule surrounding them. In this respect they were slightly larger than the parasites within the cells, the majority of which were between 1.8μ and 2.7μ, although infrequently larger cells measuring up to 3.6μ could be found. Within the parasite was a dark staining chromatin-like mass placed either at the larger end or in a semilunar arrangement at the end and along the side walls of the cells.

On the same day that this finding was made, a third specimen of blood was taken in citrate solution. When this was cultivated on Sabouraud's dextrose agar plates, fungi again were isolated.

Subcultures of the first blood culture showing growth were made in duplicate on Sabouraud's dextrose agar, dextrose extract agar, brain veal agar, and sealed brain veal blood agar slants. One set of slants was incubated at 37° C. and the other set at room temperature. Growth first appeared in the subcultures on Sabouraud's dextrose agar and dextrose extract agar kept at room temperature. Within five to six days small white colonies with a downy surface were visible. They slowly increased in size, with the production of white, feathery, aerial hyphae, arising from a base which became brown in color as the colony aged. The colonies adhered tenaciously to the surface of the media and were removed with difficulty. Similarly growth appeared on the plain brain veal agar slightly later, but at no time was growth on this media as heavy or as rapid as that on media containing dextrose.

Wet preparations of material from these colonies showed branched, hyaline, septate mycelium, 2μ to 5μ in diameter, within which were seen many dark, dancing granules. Developing arthrospores, 8μ to 13μ in diameter, were visible. They occurred on the ends of branches or from stalks along the hyphae. These smooth-walled spores also contained numerous sporelike bodies which Moore[14] considered to be endospores. The studies of Howell[15] have shown these larger cells to be true aleurospores. Occasionally in old cultures one could see tuberculate spores, pyriform in shape.

On sealed blood agar slants and in semisolid culture media containing fresh blood incubated at 37° C. the tendency was for the fungus to grow in the yeastlike stage. Cells somewhat larger than those seen in the tissues formed. These observations were similar to those of DeMonbreun,[8] who first determined the two forms of growth for this organism. The first, the yeastlike form, occurs in the body and on blood agar slants. He used the growth from these slants to reproduce the disease experimentally in monkeys. The second, the mycelial stage that we have described above, occurs on media incubated aerobically at room temperature. Intermingled with the yeastlike forms were larger cells from which hyphae appeared to be developing. The gross appearance of the growth on sealed blood agar slants was that of dull, grayish-yellow colonies, adhering well to the media. The surface of the colonies was smooth with slight tendency to the formation of aerial hyphae.

PRELIMINARY ANIMAL EXPERIMENTS

Mice. Ten mice were inoculated intraperitoneally with amounts varying from 0.5 c.c. to 1.0 c.c. of a saline suspension of the six days' growth from the sealed blood agar slants. Although there was some indication of infection in two mice, the results with these animals were unsatisfactory, perhaps due to their relative insusceptibility to infection with the fungus.

Rabbits. Two rabbits were injected intravenously with 1.5 c.c. each of a similar suspension to that used in mice. Seven weeks later the animals were killed and autopsied. The organs were normal on gross appearance. Smears prepared from various tissues failed to show the *Histoplasma capsulatum* bodies.

Guinea pigs. One guinea pig was inoculated intraperitoneally with 1.0 c.c. of the original growth in the blood culture media. This culture had been incubated for two weeks at the time it was used for the animal inoculation. The pig gradually lost weight, and at the end of seven weeks was greatly emaciated. It was killed at this time and examined for evidence of infection. Lesions typical of those found in histoplasmosis were present.

There was a collection of fluid in both the peritoneal and pleural cavities. The spleen was enlarged to approximately three times its normal size. The inguinal lymph nodes were enlarged. The lesions in the lung partially involved the pleura and gave rise to a local fibrinous pleuritis. Smears made from the peritoneal fluid, spleen, lymph nodes, lungs, liver, and bone marrow showed many histoplasma bodies present both free and within the cells.

Histologic examination. Small granulomas were seen in the lungs, liver, and spleen. *Histoplasma capsulatum* organisms were noted in these organs and also in the lymph nodes and bone marrow. The lesion was essentially similar to that described in the patient.

Histoplasma capsulatum was grown from tissue taken from the lungs, spleen, and lymph nodes.

COMMENT

The hematologic aspects of our case are of particular interest, for if the diagnosis is to be made before autopsy it must be from a study of the blood and blood culture. Once one has seen these large, vacuolated endothelial phagocytes with wavy, pale staining, light blue cytoplasm engulfing numbers of the parasites, one should be able to recognize them thereafter without difficulty. The disease may be more common than has been supposed, and the finding of an anemia with leucopenia in a weakened, emaciated patient who is found to have a septic temperature should lead one to search the blood smear for these parasitic-laden monocytes.

Presence of the *Histoplasma capsulatum* in the neutrophiles has not been noted in other cases reported. In the terminal phase these were noted in increasing numbers, the polymorphonuclear cells containing from one to five parasites. The further observation that the organisms, as in kala-azar and malaria, appear in great numbers in the bone marrow makes sternal puncture valuable as a diagnostic approach in suggestive cases.

The stage of the disease in which the parasite may first be found in the blood in human beings is not at present known. As sternal puncture, performed the

same day the organism was found in the blood only after prolonged search, showed parasites in the bone marrow in great numbers, this should prove a valuable aid in early diagnosis. Blood culture at this time also was positive.

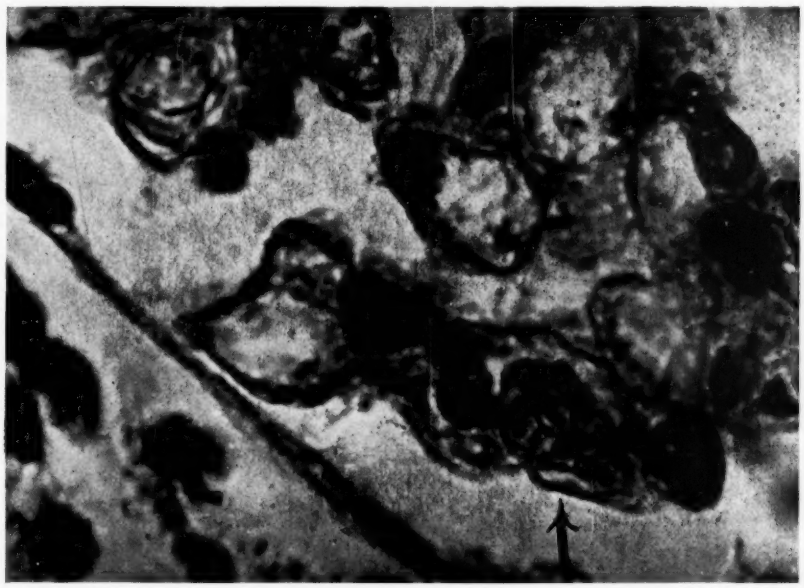


Fig. 11.—*Histoplasma capsulatum* organism in an endothelial cell in a glomerulus of the kidney (×2,500).

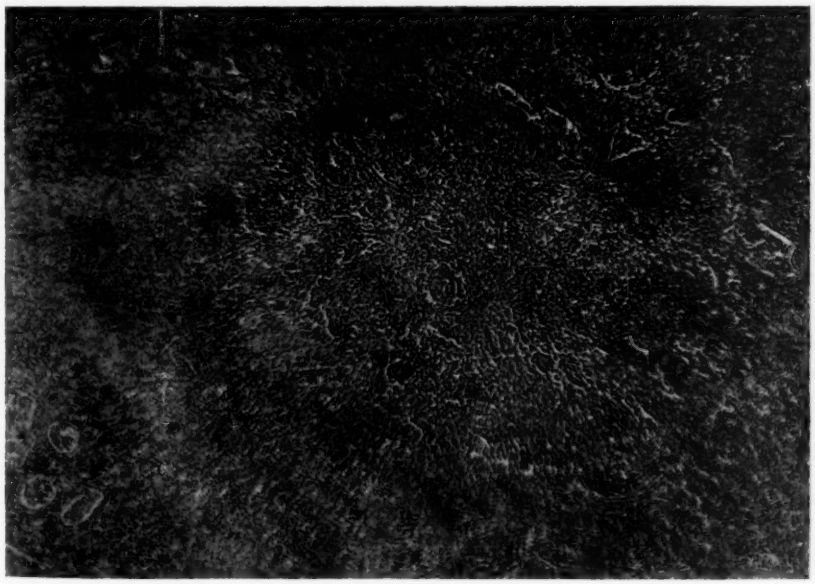


Fig. 12.—Large tubercle-like granuloma in the lung (×25).

Dodd and Tompkins[7] felt that the anemia of histoplasmosis was to be attributed in part to the phagocytosis of red blood cells. While phagocytosis must undoubtedly play a part, the leuco-erythroblastic type of blood smear and the numbers of large monocytes found in the bone marrow would suggest

that the anemia is largely due to crowding of the marrow by numbers of these large monocytes.

The tubercle-like lesions with hemorrhage in the fundi have not been noted in the other reported cases. It is unfortunate that post-mortem verification of these lesions was not possible.

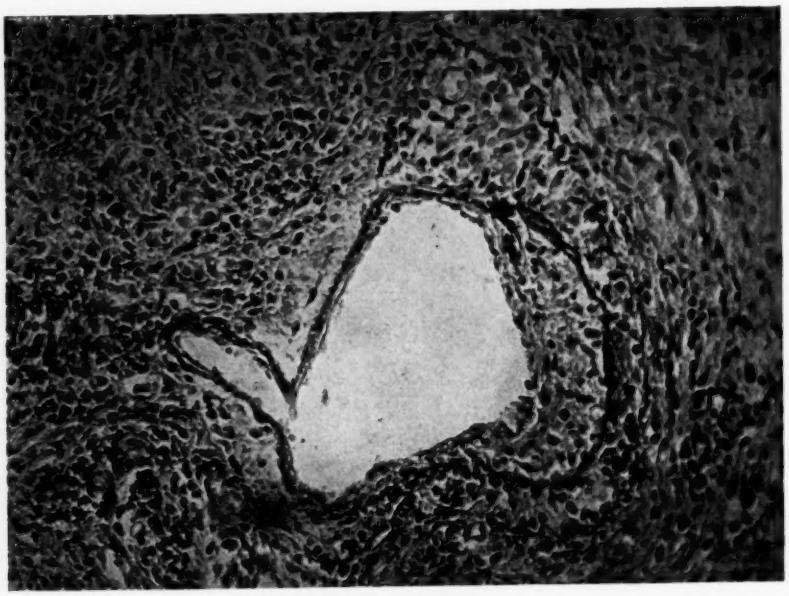


Fig. 13.—Elastic tissue stain, lung. Granulation tissue breaking through the wall producing subintimal proliferation (×150).

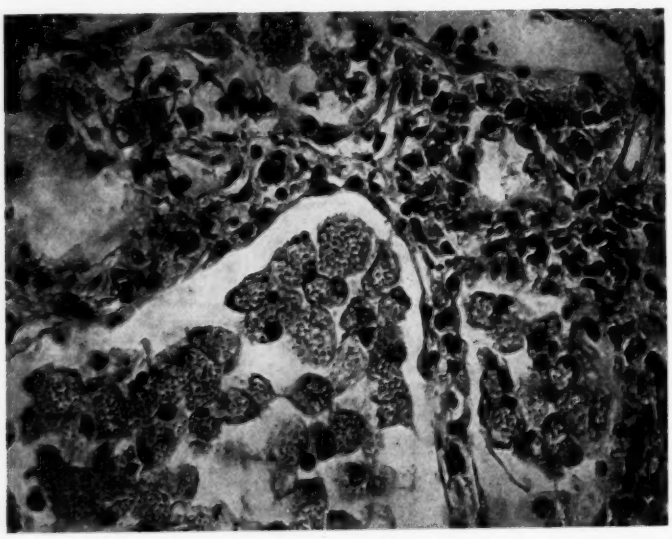


Fig. 14.—Alveoli of the lung containing large endothelial cells filled with the parasites (×360).

Histoplasma capsulatum can be grown with comparative ease on a variety of media, using either fresh or citrated blood from the patient. However, flasks of liquid or semisolid media, such as we have used, are most desirable because of the larger amounts of blood that can be inoculated.

Initial growth is slow in the blood culture media incubated at 37° C. The yeastlike forms that develop under these conditions may not be numerous enough to be easily seen in wet mounts or stained preparations until one or two weeks have elapsed. When stained by Wright's method, the young cells are in appearance very similar to those seen in blood smears from the patient. The dark staining chromatin mass within the cells lies either at the end or along the side walls. Many cells are surrounded by a clear capsular-like area.

The vegetative form of the fungus develops when these cells are subcultured on solid media and incubated at room temperature. Growth occurs more rapidly than in the initial isolation and may be visible within five to six days as small, white, downy colonies. This type of growth is characterized by the formation of large, tuberculate spores which are an aid in identifying this and related species and may be seen in wet mounts of the colonies. They have been likened in appearance by DeMonbreun[8] "to the ancient Teutonic war clubs."

No previous report of the reproduction of systemic disease in guinea pigs has been noted. We have successfully infected this animal by intraperitoneal injection of the yeastlike form. A slowly progressive infection was produced, and at autopsy the typical pathology of histoplasmosis was observed.

Thus far, our attempts to reproduce the disease in mice and rabbits have not been successful.

It is interesting to note that the parasite was seen at autopsy in the kidneys of both the patient and the experimentally infected animal. This offers the future possibility of isolating the organism from the urine. Preliminary experiments with guinea pigs, which will be reported later, bear out this suggestion. We have been able to isolate the organism from this source on several occasions.

Frequently the lesions found at autopsy have been compared with the tubercle. Though the foci vary to a certain extent in various organs according to the stage of development, they differ essentially from the tuberculous lesion in that true epithelioid cells and Langhans' giant cells are absent. The large mononuclear cell reaction in some areas may closely resemble epithelioid cells of an early tubercle, but parallel arrangement in radiating fashion, elongation to spindle-shaped cells, and a resemblance to endothelial or connective tissue elements could nowhere be demonstrated. Furthermore, the lesions of the lung differ essentially from the formation of tubercles in that the tissue undergoes necrosis apparently before proliferative reaction has commenced. The areas of necrosis in contradistinction to tuberculosis show the shadowy outline of the underlying structure and the intact vessels traversing the foci.

The involvement of vessels is a conspicuous feature, particularly the periendophlebitis, though this process is not in itself pathognomonic. It has been suggested by Phelps and Mallory,[5] and later by Dodd and Tompkins,[7] that the packing of blood capillaries and sinusoids with leucocytes causes the focal necrosis by ischemia. It is our impression that some toxic process liberated by the organism is responsible for the necrosis, since small vessels and capillaries are abundant and patent, both surrounding the nodular granuloma and even passing through the necrotic areas.

The appearance of the healing stage of the nodular granulomas found in the lung has not yet been described satisfactorily. It is uncertain in our mind

whether the nodules in our case resembling old healed tubercles should be interpreted as healing *Histoplasma capsulatum* granulomas or true tubercles. Finally, it should be mentioned that marked polymorphonuclear cell reaction with abscess formation occurs. This type of cellular reaction has not been described in any other previous case.

SUMMARY

A case of systemic histoplasmosis is reported in which diagnosis was made before death by means of smear and culture of the organism from the blood.

Experimental production of the disease in guinea pigs is described.

A detailed description of the pathologic morphology and its differential diagnosis is given.

REFERENCES

1. Darling, S. T.: A Protozoan General Infection Producing Pseudotubercles in the Lungs and Focal Necrosis in the Liver, Spleen, and Lymph Nodes, J. A. M. A. **46**: 1283, 1906.
2. Darling, S. T.: Histoplasmosis: A Fatal Infectious Disease Resembling Kala-Azar Found Among Natives of Tropical America, Arch. Int. Med. **2**: 107, 1908.
3. Riley, W., and Watson, C. J.: Histoplasmosis of Darling With Report of a Case Originating in Minnesota, Am. J. Trop. Med. **6**: 271, 1926.
4. Watson, C. J., and Riley, W. A.: A Case of Darling's Histoplasmosis Originating in Minnesota, Arch. Path. **1**: 662, 1926.
5. Phelps, B. M., and Mallory, F. B.: Toxic Cirrhosis and Primary Liver Cell Carcinoma Complicated by Histoplasmosis of the Lung, United Fruit Company, Medical Department, Fifteenth Annual Report, p. 115, 1926.
6. Crumrine, R. M., and Kessel, J. F.: Histoplasmosis (Darling) Without Splenomegaly, Am. J. Trop. Med. **11**: 435, 1931.
7. Dodd, K., and Tompkins, E. H.: A Case of Histoplasmosis of Darling in an Infant, Am. J. Trop. Med. **14**: 127, 1934.
8. DeMonbreun, W. A.: The Cultivation and Cultural Characteristics of Darling's Histoplasma capsulatum, Am. J. Trop. Med. **14**: 93, 1934.
9. Agress, H., and Gray, S. H.: Histoplasmosis and Reticulo-endothelial Hyperplasia, Am. J. Dis. Child. **57**: 573, 1939.
10. Amolsch, A. L., and Wax, J. H.: Histoplasmosis in Infancy, Am. J. Path. **15**: 477, 1939.
11. Shaffer, F. J., Shaul, J. F., and Mitchell, R. H.: Histoplasmosis of Darling, J. A. M. A. **113**: 484, 1939.
12. Clemens, H. H., and Barnes, M. L.: Histoplasmosis of Darling, South. M. J. **33**: 11, 1940.
13. Darling, S. T.: The Morphology of the Parasite (Histoplasma capsulatum) and the Lesions of Histoplasmosis, A Fatal Disease of Tropical America, J. Exper. Med. **11**: 515, 1909.
14. Moore, M.: A Morphological and Physiological Study of Two Species of Posandasia, P. Capsulata (Darling) Moore and P. Pyriformis Moore, Ann. Missouri Bot. Gard. **22**: 335, 1935.
15. Howell, A. J.: Studies on Histoplasma capsulatum and Similar Form Species. I. Morphology and Development, Mycologia **31**: 191, 1939.

THE PATHOLOGY OF ATROPHIC ARTHRITIS*

A Correlated Clinical and Laboratory Study

Charles LeRoy Steinberg, B.S., M.D., Rochester, N. Y.

THE soft and hard tissue changes which take place in rheumatoid or atrophic arthritis proceed in a certain sequence. The swelling, the increase in local heat, and redness of the soft tissues of the joint structure which the clinician observes, and the pain and stiffness of the joints which the patient notices are caused by an active inflammation of the synovial membrane, the capsule, and the surrounding soft tissue structures of the joint. The synovial membrane or stratum synoviale is normally made up of a thin layer of cells richly supplied with nuclei and a subjacent area of connective tissue cells poorly supplied with nuclei. The synovial membrane is richly supplied by a close network of blood vessels and nerves. Fig. 1 is a photomicrograph through normal synovial membrane. In atrophic arthritis there is marked proliferation of the cells of the synovial membrane which eventually produces a layer of granulation tissue or pannus over the articular cartilage. A similar process occurs simultaneously, involving the connective tissue elements of the marrow below the articular cartilage. The latter process extends through the zone of provisional calcification and destroys the articular cartilage from below, while a similar pathologic process evidenced by the proliferated synovial membrane destroys the articular cartilaginous surface. These two opposing processes eventually destroy the articular cartilage. These two layers of active granulation tissue have marked potentialities of forming fibrous and bony tissues, and when they do we have a resulting fibrous or bony ankylosis.

The synovial fluid is increased in the earlier stages and decreased in amount or completely absent in the later stages. Round cell infiltration occurs into the capsule, new blood vessels appear, and fibroblastic activity occurs. Decalcification occurs in the juxtaarticular position. This leads to rarefaction and loss of density. The pathologic picture given so far is essentially in agreement with that given by Nichols and Richardson,[1] Allison and Ghormley,[2] and others.[3] Allison and Ghormley[2] believe that atrophic arthritis has a specific micropathology. "Histologically the tissue shows a definite picture, it is as clear-cut as is that of tuberculosis, mainly a proliferative change in the synovial membrane and marrow which is characterized by focal collection of lymphocytes. This microscopic picture will, we believe, be enough to establish the diagnosis of proliferative arthritis, and in our own experience has been enough to predict the subsequent changes in several instances where the tissues showed the character-

*From the Arthritic Clinic, Rochester General Hospital, Rochester, N. Y.
Presented by title at the Seventh Annual Meeting of the American Rheumatism Association, New York City, June 10, 1940.
Received for publication, November 13, 1940.

istic changes in the early stages of the clinical disturbances." They emphasize that the focal collections of lymphocytes are not perivascular. Miller[4] adds to this interpretation. He believes that the focal collection of lymphocytes indicates that atrophic arthritis is an inflammatory disease of bacterial origin.

In 1929 Fisher[5] described a histologic picture in atrophic arthritis in which there was perivascular round cell infiltration. Parker and Keefer[6] have recently written an excellent article on the pathologic changes in three cases of atrophic arthritis in which they described the microscopic picture in some regions as showing collections of round cells which were perivascular, as described by Fisher[5] in 1929, and in other regions in which the focal collection of lymphocytes was not perivascular, as described by Allison and Ghormley[2] in 1931. These authors failed to give clinical histories in two of these cases. Such a correlated study would have added to the value of their report. Boyd[7] states that the arteries in the surrounding tissue of the diseased process in atrophic arthritis often show an obliterating arteritis and deposits of fibrin under the endothelium. This vascular change in atrophic arthritis is not mentioned in the other articles previously cited.

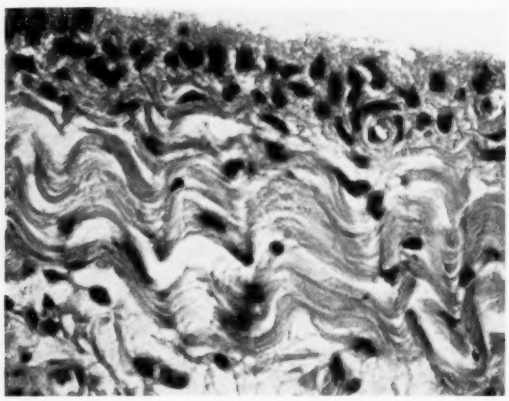


Fig. 1.—A high-power photomicrograph through normal synovial membrane. Note the lining layer of epithelial-like cells and the adjacent layer of connective tissue.

The philosopher Friedrich Nietzsche, who lived in the latter part of the nineteenth century, in his apophthegms and interludes stated, "Everything absolute belongs to pathology." And so it is. In a protean disease, such as atrophic arthritis, which is characterized clinically by remissions and exacerbations, whose onset may be acute or may be quite gradual; which may attack the young, the middle aged, and the aged; which may deform in a very short time, or else may continue in a mild course for many years, one should find different gross and microscopic pathologic findings depending upon the time in which the disease was studied, perhaps the grade of the disease, and the duration of the disease in that particular individual. I have taken case histories of various grades and durations of atrophic arthritis and correlated these with the microscopic pathologic picture to be found in that particular case. It is hoped from this unbiased report that the controversial issues of the pathologic picture of atrophic arthritis may be clarified to some extent.

CASE REPORTS

CASE 1.—L. G., a white female, aged 60 years, was first seen on April 8, 1935. She complained of painful, swollen stiff joints. She had not been able to get out of her bed for a period of several months due to crippling because of this condition. The onset of her joint trouble began in 1902, with pain and swelling of the proximal interphalangeal joints of both hands. The condition had slowly progressed until it involved practically all the joints. There was a history of two severe flare-ups, one in 1923 and one in 1933. During

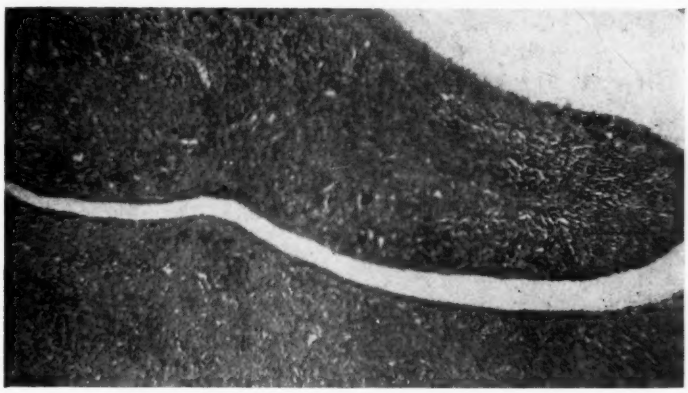


Fig. 2A.—A low-power photomicrograph showing a villous made up of proliferative synovial tissue in advanced atrophic arthritis.

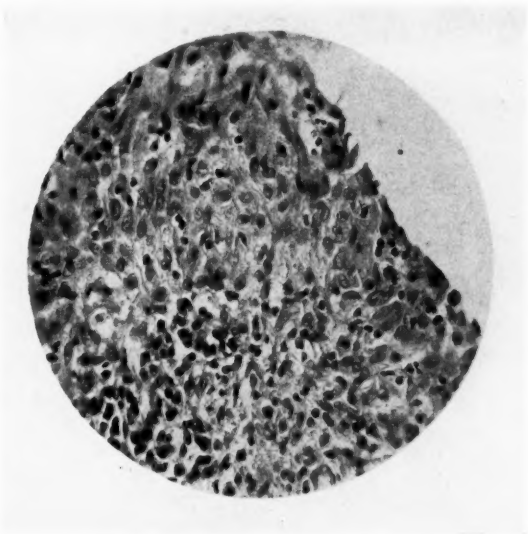


Fig. 2B.—A high-power photomicrograph of the same case showing the marked increase in fibroblasts characteristic of the "healed" or "burnt out" case of atrophic arthritis.

this period of thirty-one years most joints of the body had become involved with pain, swelling, and redness. The examination of the joints on April 8, 1935, revealed little motion in the finger joints, soft tissue swelling of the metacarpophalangeal joints with wasting of the interossei group of muscles, marked limitation of pronation and supination in the elbow joints, and little motion in the shoulder joints. Calcified tissue was present in the left olecranon bursa. Both the knee joints were swollen, and there was marked increase thickness of the capsule. Thirty degrees flexion deformity was present in both knees. The ankles were also swollen and quite tender. Blood pressure was systolic 120 and diastolic 80. Lab-

oratory examination revealed a sedimentation rate of 1.00 and a negative Wassermann test. The diagnosis was advanced atrophic arthritis. The patient was treated with bed rest, moist heat to the joints, massage, high caloric diet, and hemolytic streptococcal vaccine. The arthritic process quieted down after two months' treatment. A right synovectomy was done on June 14, 1935. There was marked increase in fluid in the joint, extensive arborescent villous growth, and marked destruction of the cartillage. Microscopic sections of the synovial membrane removed from this joint are shown in Figs. 2*A* and 2*B*. One is a low-power

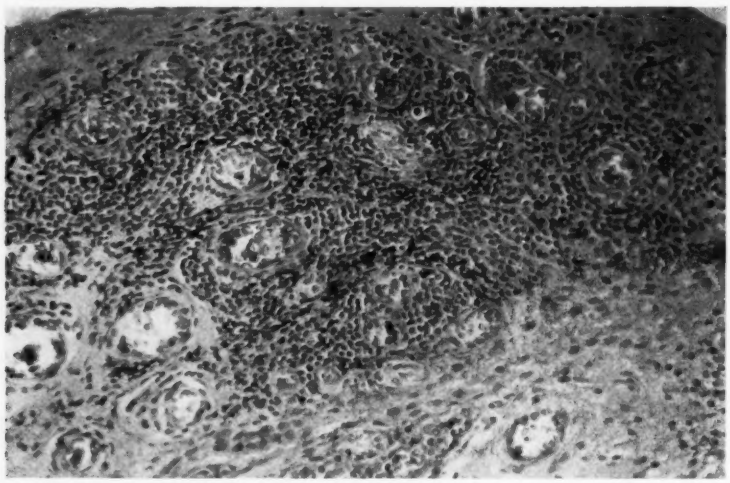


Fig. 3*A*.—A low-power photomicrograph showing a focal collection of lymphocytes, increase in capillaries, and in fibroblasts.

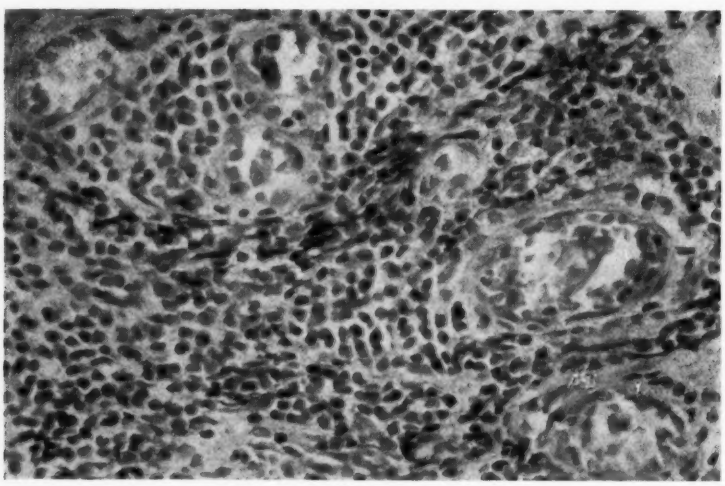


Fig. 3*B*.—A high-power photomicrograph of the same case showing a diffuse round cell rather than a perivascular round cell infiltration.

and the other is a high-power photomicrograph of this tissue. These sections show marked increase in granulation tissue and areas of hyalinization. There is also diffuse marked increase in round cell infiltration. The high-power photomicrograph particularly shows the increase in fibroblastic activity and the decrease in cellular infiltration. Cultures from the joint and guinea pig inoculation were negative. The pathologic picture in this case was typical of a chronic advanced atrophic arthritis. The marked proliferation of synovial tissue, as evidenced by the villous growth and the replacement of the synovial tissue by fibroblasts and the relative absence of lymphocytes in the synovial membrane in this case, represents the so-called "healed" type or "burnt out" case.

CASE 2.—C. N. H., a white female, aged 47 years, was first seen in February, 1938, complaining of pain and swelling and limitation of motion of the right knee of one month duration. The blood pressure was systolic 120 and diastolic 85. Examination of the right knee revealed diffuse swelling and moderate tenderness of the joint. There was little limitation in motion of this joint. A synovectomy was done, and section through the synovial membrane revealed the so-called typical focal collection of lymphocytes, as described by Allison and Ghormley,[2] increase in fibroblastic activity, and increase in the number of capillaries (Fig. 3*A*). Culture from the joint and guinea pig inoculation were negative. The high-power photomicrograph of this tissue definitely reveals the absence of perivascular round cell infiltration (Fig. 3*B*).

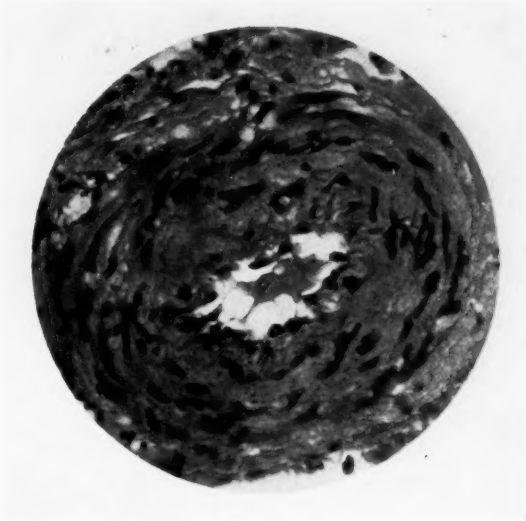


Fig. 4.—A photomicrograph through a blood vessel of the synovial membrane in a case of atrophic and hypertrophic arthritis complicated by essential hypertension and syphilis.

CASE 3.—H. B., a white female, 54 years of age was first seen on July, 1938, complaining of a painful swollen right knee of ten months' duration. Examination of the joint revealed moderate swelling of both joints. An x-ray examination revealed evidence of hypertrophic arthritis as evidenced by new bone production. The blood pressure was systolic 240 and diastolic 118. The patient was a known syphilitic who had been treated for syphilis for five continuous years up until one year before the present observation. Her last Wassermann test was minus, 3 plus, 3 plus. A section through a blood vessel of the synovial membrane of the right knee is shown in photomicrograph Fig. 4. The marked increase in thickness of the muscular wall may be due to the hypertension. The fibrinous exudate in the lumen of the vessel may be due to the syphilitic process. This case emphasizes the need of correlated clinical and pathologic study before conclusions as to the exact pathology of atrophic arthritis are made.

CASE 4.—S. M., a white female, aged 27 years, gave a history of one year's duration of painful swollen joints. The patient first noticed pain and swelling in the smaller articulations, but this gradually progressed until most joints of the body had become involved. She had noticed definite flare-ups associated with upper respiratory infections. The physical examination revealed a well-developed, but poorly nourished, white female. Her tonsils had been removed, and the dental examination both on physical and by radiographic examinations were negative. The posterior pharynx appeared moderately injected. Vaginal examination revealed the patient to be a virgin. Examination of the joints revealed the following: Marked limitation of motion of the right wrist and finger joints; a swollen, inflamed, and quite tender, right knee. Laboratory examination: Sedimentation rate on November 30, 1936, 1.10; serum calcium 11.3; serum phosphorus 4.5; Wassermann reaction negative. Throat

culture revealed both hemolytic streptococci and *Streptococcus viridans*. Vaginal smear and culture were negative. The patient was treated with bed rest, high caloric diet, moist heat, and hemolytic streptococcal vaccine. The sedimentation rate on December 21, 1936, was 0.88. Laboratory examination on January 4, 1937, revealed a sedimentation rate of 0.91, a negative complement fixation test for the gonococcus, a basal metabolism minus 1 per cent; throat culture was negative for hemolytic streptococci. X-ray examination of the joints revealed a negative examination as regards bone structure of the left knee. There was slight narrowing of the joint spaces of the temporomandibular joints. X-ray examination of the right knee on September 16, 1937, was no longer normal. There was roughening of the spine of the tibia, roughening of the joint surface, particularly the lateral aspect.

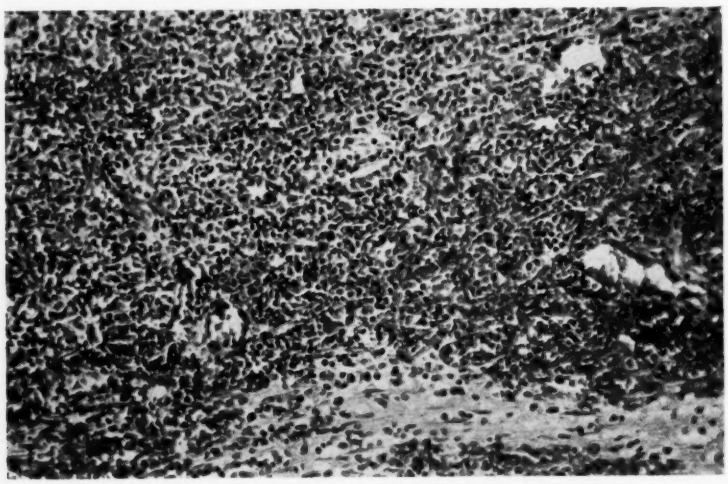


Fig. 5*A*.—A low-power photomicrograph through the synovial membrane of case S.M. showing both polymorphonuclear and round cell infiltration.

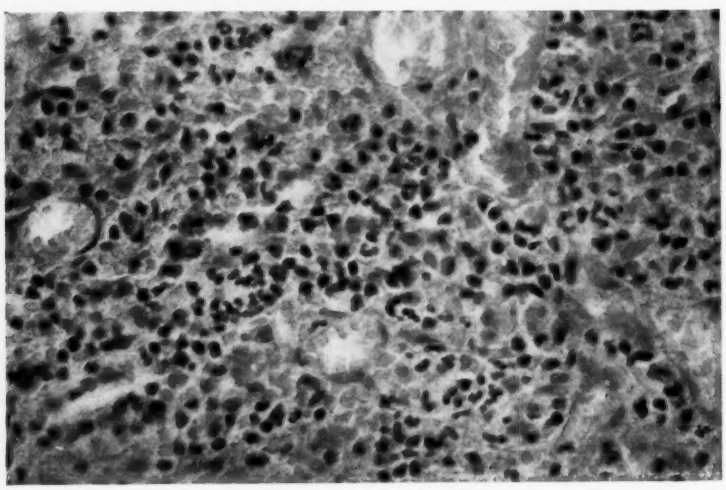


Fig. 5*B*.—A high-power photomicrograph through the same tissue showing the "purulent" inflammatory reaction characterized by the polymorphonuclear infiltration.

There was also questionable narrowing of the joint space. Both knees and the left ankle were markedly swollen, and there was definite increase in local temperature on January 13, 1938. The sedimentation rate at this examination was 1.53. The patient was treated at home by bed rest, hot baths, general massage, high caloric diet, and hemolytic streptococcal vaccine until November 14, 1938. She had shown marked clinical improvement during this

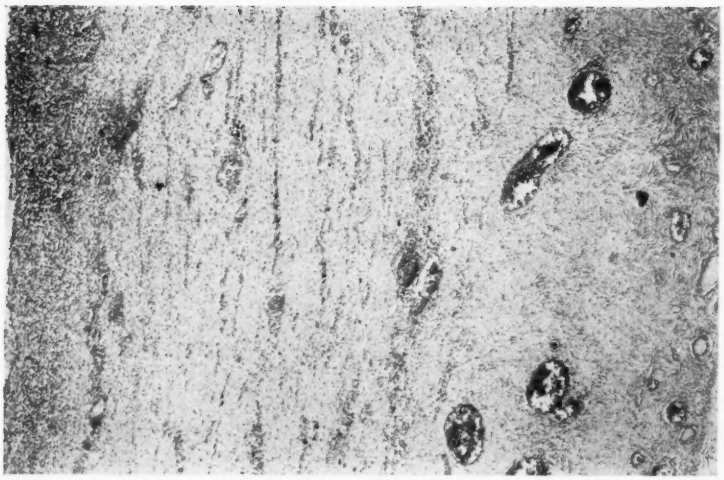


Fig. 6*A*.—A very low-power photomicrograph through the entire synovial membrane removed from the left knee of case S.M. This shows diffuse round cell infiltration of the entire layer, most pronounced near the surface.

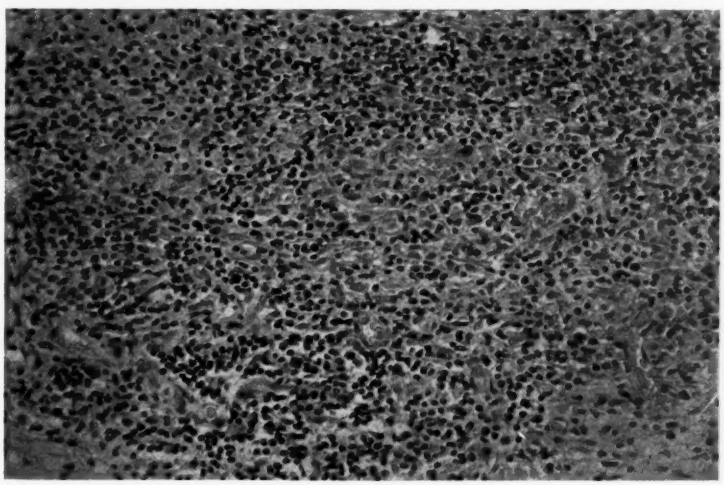


Fig. 6*B*.—A high-power photomicrograph through the surface layer of case S.M. showing marked round cell infiltration.

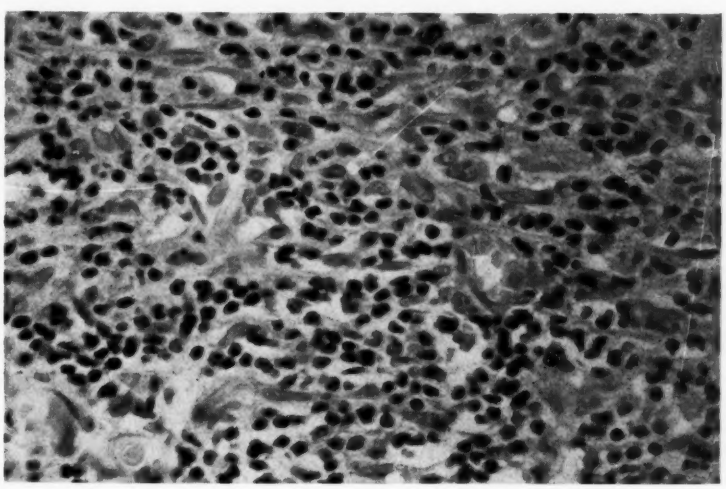


Fig. 6*C*.—A high-power photomicrograph through the synovial membrane of case S.M. showing diffuse round cell infiltration and increased fibroblastic activity.

period, and it was deemed advisable to do a bilateral synovectomy. A right synovectomy was done on November 21, 1938. Figs. 5 and 5*A* are sections through the synovial membrane removed from the right knee during this operation. Fig. 5*A* is a low-power photomicrograph, and Fig. 5*B* is a high-power photomicrograph. The most striking feature is the presence of polymorphonuclear cells throughout the membrane. In fact, the synovial membrane is much fibrosed, and the inner layer showed a purulent inflammatory reaction, a finding much more common in typical pyogenic arthritis than in atrophic arthritis. The patient made an uneventful recovery, and a synovectomy was done on the left knee on December 14, 1938. Figs. 6*A*, 6*B*, 6*C*, and 6*D* are sections through the synovial membrane removed from this knee joint. Fig. 6*A* is a very low-power photomicrograph, and Figs. 6*B* and 6*C* are high-power photomicrographs. The inner surface of this synovial membrane showed much fibrosis and very marked infiltration of the round cells. This round cell infiltration was diffuse. Fig. 6*C* is a section through the synovial membrane removed from the left knee; it shows definitely that the round cell infiltration is not perivascular. Fig. 6*D*, a very high-power photomicrograph of another section through the same synovial layer, showed a blood vessel with definite perivascular round cell infiltration.

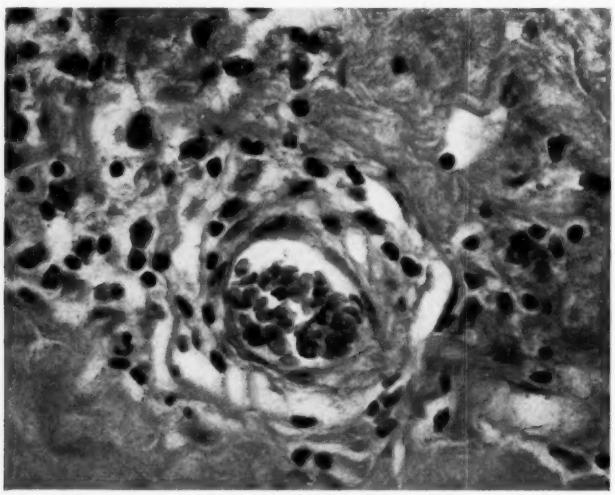


Fig. 6*D*.—A very high-power photomicrograph taken through another area of the synovial membrane in case S.M. showing perivascular round cell infiltration.

COMMENT

No specific micropathology exists in chronic atrophic arthritis. The disease is characterized by remissions and exacerbations. It is ushered in rather acutely in some cases and there is marked destruction of the joints in a comparatively short period of time, while in others the disease is more or less quiescent for a period of years. I have seen some cases in which chronic atrophic arthritis has been present for thirty years with no resultant deformities in the joints. I have seen others in which the patient has been made a cripple in the relatively short space of six to eight months. Perhaps it is this protean nature of the disease that accounts for the different pathologic pictures. It is hoped that the photomicrographs shown in this article demonstrate most of the changes that occur in the synovial layer in atrophic arthritis. The picture varies from one in which there is polymorphonuclear infiltration (this section being obtained in a joint which was quite active at the time of operation) to

sections showing round cell infiltration, both perivascular and nonperivascular. A "burnt out" case showing the process after many years of involvement, with little round cell infiltration but replacement of the synovial tissue by means of scar tissue, is shown. It must also be remembered that atrophic arthritis is both a common and a chronic disease, and that, therefore, other diseases may be associated with this condition and may tend to give a varied pathologic picture. Conditions such as syphilis and hypertension, and other diseased conditions, which may affect the blood vessels of the body, must be remembered, since they must be ruled out before concluding that the pathologic picture found in the synovial tissue is the one that is characteristic of atrophic arthritis. It is hoped that correlated clinical and pathologic studies, even though they be a few cases, may offer much in the elucidation of the processes involved in atrophic arthritis.

REFERENCES

1. Nichols, E. H., and Richardson, F. L.: Arthritis Deformans, J. M. Research **21**: 149, 1909.
2. Allison, N., and Ghormley, Ralph K.: Diagnosis in Joint Disease, New York, 1931, William Wood & Company.
3. Report of the British Medical Association, Committee on Causation and Treatment of Arthritis and Allied Conditions, Brit. M. J. **1**: 1033, 1933.
4. Miller, J. L.: A Critical Review of the Literature on Chronic Rheumatism, Arch. Int. Med. **57**: 213, 1936.
5. Fisher, A. G. T.: Chronic Arthritis, New York, 1929, Macmillan Co., p. 34.
6. Parker, F., Jr., and Keefer, C. S.: Gross and Histologic Changes in the Knee Joint in Rheumatoid Arthritis, Arch. Path. **20**: 507, 1935.
7. Boyd, W.: A Textbook of Pathology, Philadelphia, 1938, Lea & Febiger, p. 1011.

SODIUM SULFAPYRIDINE MONOHYDRATE INTRAVENOUSLY IN THE TREATMENT OF LOBAR PNEUMONIA*

ABEL LEVITT, M.D., F.A.C.P., HAROLD T. SCHWEITZER, M.D., AND KENNETH GOLDSTEIN, M.D., BUFFALO, N. Y.

CHEMOTHERAPY in the treatment of pneumonia has undergone considerable change in recent years. As a result of the introduction of sulfapyridine in the treatment of the disease, the mortality rate has markedly declined. In 1939 we reported the results of treatment of 100 consecutive cases of lobar pneumonia by oral administration of sulfapyridine. Our mortality rate in that group was 3 per cent. We observed no serious reactions to the drug, although there were some mild toxic manifestations. Occasionally the condition of the pneumonia patient is such that the oral use of this essential therapeutic agent is impossible because of nausea, vomiting, delirium, and other complications. Here the parenteral use of the drug becomes necessary. On the Medical Service of the Edward J. Meyer Memorial Hospital, sodium sulfapyridine monohydrate

*From the Medical Service of the Edward J. Meyer Memorial Hospital and the University of Buffalo School of Medicine.

Received for publication, December 14, 1940.

was used intravenously in 25 cases in order to compare its effect when thus given to the oral administration of sulfapyridine.

In this group of cases there were about an equal number of males and females, between the ages of 20 and 70 years, the majority of them being under 50 years of age.

Table I indicates the duration of illness prior to admission to the hospital when treatment was instituted.

TABLE I

DURATION OF ILLNESS PRIOR TO HOSPITALIZATION

NO. OF DAYS	NO. OF CASES	PER CENT
0-2	17	68
3-5	5	20
6-9	2	8
10-14	0	0
Above 2 weeks	1	4

It will be noted that about one-third of the cases did not enter the hospital until the third day or more after the onset of the disease.

Sputum typing and blood cultures were done on each case, and results are recorded in Table II.

TABLE II

LABORATORY STUDIES

TYPE OF PNEUMOCOCCUS	NO. OF CASES	BLOOD CULTURE	RESULTS
I	1	Positive	Recovered
II	6	Positive in 1 case	5 recovered 1 died
III	2	Negative	Recovered
IV	3	Positive in 1 case	Recovered
VIII	2	Negative	Recovered
Undetermined	2	Negative	Recovered
Negative	9	Negative	8 recovered 1 died

This year there appeared to be quite a diversity in the pneumococcic types of pneumonia. Usually we find a more predominant type, but the virulence in each particular type varies considerably from time to time. Blood counts were done in each case daily. The majority of our cases had a leucocytosis prior to the administration of the drug. In only rare instances did we use the drug when the white blood count was less than 10,000.

Table III shows the blood counts before treatment.

TABLE III

BLOOD COUNTS

W.B.C.	NO. OF CASES	POLYS.	NO. OF CASES
0- 5,000	0	50- 60	1
5-10,000	3	60- 70	5
10-20,000	17	70- 80	6
20-30,000	4	80- 90	10
30-40,000	1	90-100	3

TABLE IV

GENERAL ANALYSIS OF BLOOD CONCENTRATION

CASE	DRUG STARTED	FIRST INJECTION (MG. % IN BLOOD)					SECOND INJECTION (MG. % IN BLOOD)					THIRD INJECTION (MG. %)			FOURTH INJECTION	DRUG STOPPED	TOTAL MG. DRUG USED	TEMP. ON ADM.	DAY OF CRISIS OR LYSIS
		10 MIN.	1 HR.	4 HR.	8 HR.	24 HR.	10 MIN.	1 HR.	4 HR.	8 HR.	24 HR.	10 MIN.	1 HR.	4 HR.	24 HR.				
H. D.	4/27/40	7.7	5.9	4.2	–	Less than 1	7.6	5.5	4.2	–	Less than 1	3.7	1.9	1	–	5/ 1/40	20	105	4th
L. P.	4/28/40	8.7	8	7.1	5.3	4.3	–	–	–	8.9	3.8	–	–	–	–	5/ 1/40	12	105	4th
C. S.	4/30/40	8.9	7.6	2.8	2	0.7	8.8	7.3	2	3.8	1	–	–	–	–	5/ 2/40	12	101.3	2nd
F. R.	5/ 6/40	7	6	5	4.5	2	12	10	11	10	2	10	–	–	–	5/11/40	20	100.6	2nd
D. S.	5/14/40	11	7	6	6	6	10	10	10	10	6	10	–	–	–	5/18/40	20	101.8	2nd
J. S.	5/14/40	10	10	7	6	7	12	12	10	10	5	4	–	–	–	5/18/40	20	102.3	3rd
L. K.	5/15/40	8	7	5	1	1	5	5	5	Trace	–	–	–	–	–	5/22/40	28	102.0	6th
C.H.	5/17/40	9	6	7	6	3	5	4	2	5	7	–	–	–	–	5/22/40	24	103.2	3rd
C. S.	5/23/40	9	5	5	4	1	10	8	7	6	4	–	–	–	–	5/26/40	16	103.6	3rd
L. T.	8/23/40	11	9	8.4	6.6	4.1	17.9	14.8	12.6	10	5.6	–	–	–	–	8/27/40	20	102.8	2nd
H. P.	7/27/40	4	4	–	3.4	3	2.2	7.4	5.8	4	3.5	2.3	–	–	–	8/ 1/40	20	105.4	8th
M. S.	7/27/40	5.9	7.5	8.6	11	9.1	10.1	9	8.3	6	1	–	–	–	10.8	7/31/40	20	102.6	3rd
N. S.	7/27/40	4.8	4	3.2	3	2	6	5.4	4	4	3	–	–	–	8	7/30/40	12	105	2nd
S. H.	6/ 2/40	2	8	5	4	5	–	–	–	–	–	–	–	–	–	6/4 /40	10	102.8	4th
M. D.	5/28/40	7	6	7	8	9	14	9	9	8	4	–	–	–	7	5/31/40	16	100.2	5th
S. P.	5/27/40	7	5	5	1	1	8	6	5	4	Trace	–	–	–	–	5/31/40	20	104.8	8th
N. H.	5/29/40	5.2	3.9	3.8	3.4	–	5.6	4.8	–	–	–	–	–	–	–	5/31/40	12	102.6	–
H. D.	10/ 6/40	10.9	9.1	13.1	6	11.4	9.1	11.3	10	9.4	9	–	–	–	–	10/13/40	24	101.8	2nd
B. H.	10/15/40	20	13.3	10	8.3	7	18	14	9	–	–	–	–	–	–	10/17/40	12	104	2nd
G. B.	9/28/40	10	7.8	6.9	5.6	13.8	10.3	8.8	7.8	–	29.6	–	–	–	–	9/30/40	12	99.4	2nd
L. N.	10/13/40	7.8	5.4	5.1	4.1	8.8	10.4	7.2	5.8	–	–	–	–	–	–	10/14/40	8	101	2nd
J. R.	10/18/40	8	6.7	4.6	–	10.7	7.6	7.0	7.6	–	–	–	–	–	–	10/20/40	12	103	4th
R.T.	10/18/40	4.2	3.8	3.3	–	5.9	6.7	3.1	5.9	7.6	–	–	–	–	–	10/21/40	16	101	2nd
H. F.	10/17/40	6.3	4	2	–	–	3	4.6	8	4	–	–	–	–	–	10/21/40	20	103	4th
R. B.	10/15/40	8	5.3	4	5	4.3	–	–	7	4	–	–	–	–	–	10/20/40	25	101	3rd

METHOD OF ADMINISTRATION OF SODIUM SULFAPYRIDINE MONOHYDRATE

After the diagnosis of lobar pneumonia had been made and corroborated by x-ray, each patient was placed on a liquid diet containing a minimum of three liters per day, made up of fruit juices and milk. If oral fluids were not taken in sufficient quantity, we resorted to parenteral administration of saline and glucose. The only other routine therapeutic agent used was an opiate if indicated for cough or sedation, and in some instances oxygen by nasal catheter. The latter, however, did not seem to relieve the cyanosis when present while this drug was being used.

The sodium sulfapyridine was administered slowly intravenously, using a 5 per cent solution (4 Gm. of the powder dissolved in 80 c.c. of distilled water). Eighty cubic centimeters were given daily, and the blood was examined for per cent of sulfapyridine content at frequent intervals during the twenty-four hours. In rare instances the dose was repeated before the twenty-four hours were up in order to maintain a higher blood level. We did not supplement this therapy with the oral use of the drug, since we desired to determine its effect by the intravenous method only.

TABLE V

REACTIONS

REACTION	INTRAVENOUS 25 CASES (%)	ORAL 100 CASES (%)
Nausea and emesis	84	17
Cyanosis	52	18
Headache	36	None
Delirium	16	4
Jaundice	12	None

By this method of administration when a single dose was given daily, irrespective of size of the patient, the per cent of sulfapyridine in the blood shortly following injection (ten minutes) reached an average level of 8 mg. per cent and dropped about 20 per cent after an interval of one hour. Determinations at the end of four hours showed an additional 5 per cent drop. At the eighth hour there was an average decrease in sulfapyridine of 37.5 per cent. Recording of blood level for the drug at the end of twenty-four hours showed no appreciable change from the eight-hour level, the average being about 5 mg. per cent. Since it is advisable to maintain a blood level between 5 and 10 mg. per cent, it seems that the drug should be administered at least twice daily, and in some instances three times daily. With the above dosage the blood concentration, as noted in Table IV, varies considerably in each patient, irrespective of age, sex, or weight. When using sulfapyridine orally, the dose used was 2 Gm. on admission, followed by 1 Gm. every six hours and continued for three days following drop of temperature to normal. The blood concentration level on the average in twenty-four hours, with use of the oral drug, was 3.93 mg. per cent, while with the intravenous drug given once daily, it was about 5.6 mg. per cent. The duration of illness following the intravenous administration of the drug varied somewhat. In about 40 per cent of the cases the temperature came down by lysis or crisis within forty-eight hours. In one-third of the group this oc-

curred within four days. The remainder varied betwen five and eight days, none more than eight days. This compared closely with the data obtained with the oral use of the drug.

Reactions were similar to those obtained when sulfapyridine was used orally. Nausea and emesis were present in a large group of our cases with the intravenous use of the drug. Cyanosis was more marked, headache was present in a third of the group, jaundice (hemolytic) was observed in two cases (none in oral group), and a toxic hepatitis with regurgitant jaundice was noted in one case.

SUMMARY AND CONCLUSIONS

In this group of 25 cases we have had two deaths: one was a senile patient with hyperthyroidism who died from heart failure; the other had type II pneumonia and developed toxic hepatitis. Of significance in our group was a patient with an eight months' pregnancy who was delivered and recovered. Another had an infected abortion; she developed pneumonia and recovered. One had malignant hypertension and uremia, recovered from the pneumonia, but died some weeks later from his cardiovascular disease. We do not consider his death due to pneumonia.

The intravenous use of sodium sulfapyridine monohydrate has no particular advantage over the oral use of sulfapyridine or sulfathiazole when the latter can be taken orally by the patient. In those patients who for some reason are unable to take the drug orally, the intravenous sodium sulfapyridine should be used in conjunction with pneumonia sera when indicated. From experience, one to two injections daily of 80 c.c. of 5 per cent solution are required to maintain a blood concentration of 5 to 10 mg. per cent. If laboratory facilities are available, one could, by frequent blood examination, determine the exact frequency for the administration of the intravenous drug.

REFERENCE

1. Levitt, Abel, Levy, Dexter S., and Jaskiewicz, Stanley J.: Clinical Experience With Sulfapyridine in the Treatment of Lobar Pneumonia, New York State J. Med. 39: 1916, 1939.

333 Linwood Avenue

THE AVERAGE LENGTH OF LIFE OF THE RED CORPUSCLE*

DONA GAYLER GRAAM, A.M., PH.D., TERRE HAUTE, IND.

IN THIS study an attempt is made to add to the knowledge of the ever recurrent problem of the length of life of the red corpuscle. Hunter (1884 and 1887), and later Ashby (1919), have given a very good brief history of early investigations. Ashby (1919) also explains why, as yet, we cannot consider the problem solved. Escobar and Baldwin (1934) add somewhat to these early investigations.

Many chemists because they have perfected accurate methods for bile pigment estimation feel that they can thus determine the number of corpuscles destroyed every day and hence the number made every day if their number remains constant. Whipple (1926) criticizes this view by pointing out that there are at least two unknown factors which could seriously disturb these calculations. First, muscle hemoglobin certainly contributes to the amount of bile pigment. Second, hemoglobin set free in the circulation may be changed to bile pigment and urobilin, or it may be used again in hemoglobin construction. Miss Ashby (1919) (1921)[1, 2] has done the most acceptable work so far on this subject. She has shown that the transfused corpuscles live in a recipient's blood stream for thirty days or more. The chief objections to accepting her results as far as the length of life of the corpuscles is concerned have been discussed by the others. Wean and Warren[17] point out that what may be the length of life of a transfused corpuscle may not be the length of life of a native corpuscle. Foreign cells, especially of a different blood group, which necessarily were used by Miss Ashby, may not be as susceptible as native cells to ordinary means of destruction. A second objection is made by Rous (1923) that the "foreign" protein of the stroma of the transfused corpuscles may be utilized over and over again by the recipient and thus form corpuscles unlike the usual corpuscles of the recipient.

Eaton and Damren (1930)[5, 6] explained a method for the estimation of the average length of life of the red corpuscle which has been verified only recently by kinsmen Moore and Harrison (1940). They considered the red blood corpuscles of the blood stream as a population. In a population that remains constant, as many individuals die as are born, or vice versa. If in that population a catastrophe occurs which destroys one-third or one-fourth of the population, immediately an increased number of blood corpuscles, all approximately the same age, is thrown into the blood stream. These new individuals

*From a private clinical laboratory, Rose Dispensary Building, Terre Haute, Ind.

A preliminary report was made in a manuscript submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology of the Graduate School at Indiana University. This article represents much subsequent work.

Received for publication, December 18, 1940.

that restore the population are called reticulocytes and can be followed by using a special technique (Spohr, 1930). The new thing that Eaton and Damren (1930)[5, 6] point out is the fact that in a population where there are so many individuals of the same age, these individuals finally arrive at that age, corresponding to their average age; then a greater than the usual number die at the same time. This intravascular hemorrhage is sufficient to stimulate another noticeable reticulocyte production. They claim that the average length of life of the corpuscle is the length of time between two successive peaks of reticulocyte production. The one objection to their conclusion is that the second peak of reticulocyte production would occur *at an appropriate distance after the death of a great number of corpuscles that had lived their average length of life and that had appeared in response to the initial hemorrhage.*

By making a daily total erythrocyte count along with the daily reticulocyte count, any unusual population of old corpuscles dying at one time will show itself in the drop in erythrocyte count. This "death drop" may amount to as much as a million per cubic millimeter of blood within a period of two days, as will be shown in the subsequent description of my experiments upon the rabbit. That this sudden death of erythrocytes represents the termination of the natural length of life of the reticulocytes born in response to the previous hemorrhage can scarcely be doubted.

Definition of Reticulocytes.—Any mammalian red corpuscle showing a stippling, tiny threads, or network light or heavy when stained with brilliant cresyl blue is called, in this paper, a reticulocyte. Key (1921) explains this basophilic substance in a rather complete manner. King (1928)[12] also gives a concise statement concerning the reticulocyte and Cunningham (1920) describes them rather well.

Experimental Procedure.—Well-nourished rabbits, as determined by weight and red blood count of between six and seven millions and a high hemoglobin percentage, and of known age were kept under uniform conditions in the laboratory. The food consisted of mixed ground grain in pellet form, alfalfa hay, and crushed oats with some green food every day.

A control period of several days was run in the case of each rabbit to determine the normal variation in the percentage of reticulocytes, red corpuscles per cubic millimeter of blood, and in one case the hemoglobin per cent. If an animal had more than 2.5 per cent, that animal was discarded. The drop of blood for the daily test was withdrawn from the marginal ear vein by the puncture method, at approximately the same time each day and before the animal was fed.

The blood for the hemorrhage was removed directly from the heart by means of a hypodermic needle and without the use of an anesthetic. An amount of blood estimated to be one-fourth to one-third of the total blood, or about 1.25 per cent of the body weight, was withdrawn. The blood was withdrawn slowly so that the vasomotor system could compensate for its loss. The animal was held quietly by the assistant for a period of five or ten minutes after the hemorrhage and then put back in his hutch and fed and watered as usual.

A period of forty-eight hours after hemorrhage was allowed to pass before the daily tests were begun again. Male rabbits only were used.

Staining Technique.—I am indebted to Dr. J. A. Badertscher for the method used in staining the reticulocytes of mammalian blood. The method is simple, quick, and accurate and has not been published.

A drop of fresh blood is placed directly on a glass microscope slide, taken from the ear of a rabbit or finger of a human being. A glass rod, on the end of which is deposited brilliant cresyl blue stain,* is used to stir the blood until it is colored a uniformly greenish blue. The edge of another microscope slide is then placed in the droplet of blue-colored blood. Sufficient time is allowed for the blood to flow along the edge of the touching surface of this latter slide. Then the blood on the edge of this latter or second glass slide is spread on to a third slide by pushing it along on the surface of the third slide in the usual way of making a blood smear. As many as eight or ten blood smears can be made on as many different glass slides from the one drop of stained blood, providing the edge of a clean slide is used every time for spreading the blood on other clean slides.

As soon as the blood smear is made and before it is dry, it should be held in a moist chamber a few seconds. The mouth is a very good moist chamber, providing the breath is held while the slide is in it. This permits the corpuscles to be exposed to a saturated stain longer than otherwise, and there is less distortion of the corpuscles. The moisture of the breath, if blown on the slide, will cause hemolysis of the corpuscles. The smear dries in a minute or two after it is removed from the moist chamber and is then ready to be counterstained with Wright's stain, mounted in balsam, and examined at any subsequent time.

Counting.—In the determination of reticulocyte percentage, the cells were counted by means of an ocular Micrometer Disc. The total red blood cell count and the reticulocyte cell count for successive squares were each determined. This was continued until a thousand or more cells had been counted and then the percentage of reticulocytes was estimated.

In making the red blood corpuscle count for the total number of red corpuscles per cubic millimeter of blood, Trenner diluting pipettes and Levy-Hausser counting chambers were used.

RESULTS

Young Rabbit.—Table I and Fig. 1 present the results of an experiment on a young rabbit of from six to seven months old. The experiment lasted twenty-nine days. The conditions of the blood were quite regular during the control period of six days. The average number of red blood corpuscles per cubic millimeter of blood remained constant near 6.4 million, and the hemoglobin estimate was 92 per cent. The reticulocyte percentage fluctuated only slightly, which fluctuation could reasonably be within experimental error. The reticulo-

*A saturated solution of brilliant cresyl blue, using 100 per cent alcohol as a solvent, is used. The tip of a glass rod is dipped in the solution, and the alcohol is burned off in the flame of an alcohol lamp. The glass rod is dipped three or four times until sufficient dye is deposited on the glass rod.

cyte percentage averaged 2.4 per cent, which would make the actual number of reticulocytes present at any one time, per cubic millimeter of blood, about 153,600.

On the sixth day of the experiment after the three blood tests mentioned above had been made, the animal was bled of an estimated one-fourth to one-third of its blood, or about 40 c.c. A period of forty-eight hours was allowed to elapse in order that the liquids of the body could adjust themselves, and then the blood was studied again daily until the end of the experiment. On the eighth day of the experiment, two days after the hemorrhage, a marked change due to prehemorrhagic conditions was shown in the blood when the red corpuscle count, reticulocyte percentage, and hemoglobin percentage were taken. The red corpuscle count had fallen to 4.1 million, the hemoglobin percentage to 56, and the percentage of reticulocytes had increased to 5.1.

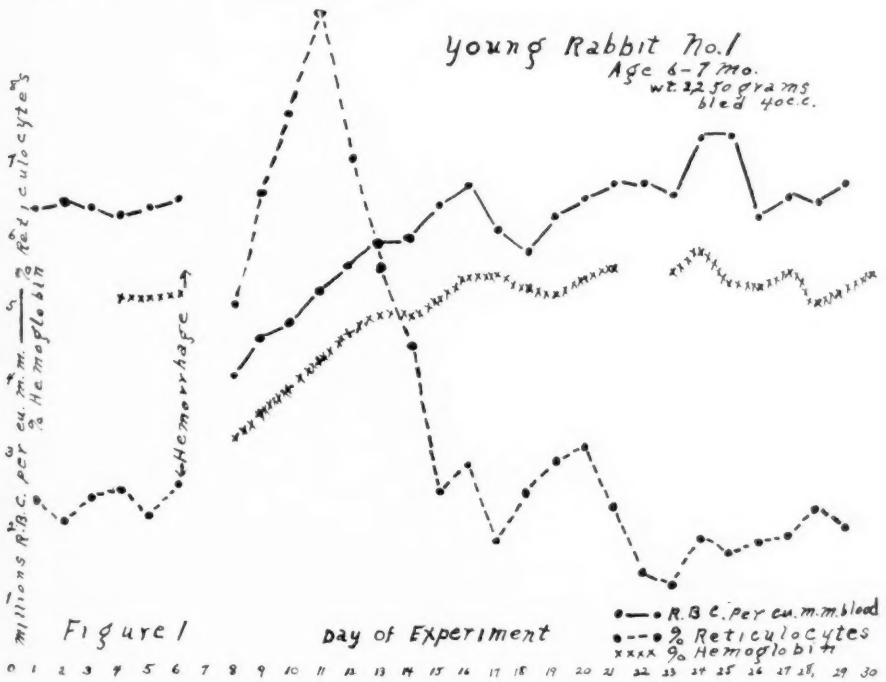

Young Rabbit No. 1
Age 6-7 mo.
wt. 2250 grams
bled 40 c.c.
Millions R.B.C. per cu. m.m.
% Hemoglobin
% Reticulocytes
Hemorrhage
Figure 1
Day of Experiment
R.B.C. per cu. m.m. blood
% Reticulocytes
% Hemoglobin
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig. 1.—The per cent of reticulocytes, the total red corpuscle count, and the hemoglobin percentage were taken before and after a hemorrhage of 40 c.c. of blood, approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Three peaks of reticulocyte formation occurred on the 11th, 20th, and 28th days of the experiment. The total red corpuscle count was lowest on the 18th and 26th days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 11th and 18th days and between the 20th and 26th days. The average length of life of the red corpuscles was 6.5 days.

The increase in reticulocytes was actual as well as relative. Although the erythrocyte number per cubic millimeter was less than two-thirds the prehemorrhagic number, the actual number of reticulocytes per cubic millimeter was 209,100 instead of the 153,600 actually present at any one time in a cubic millimeter of blood during the control period. The expected drop in the number of erythrocytes, if one-third of the blood had been removed, would have been to 4.3 million, and the actual number was approximately 4.0 million. The per-

centage of reticulocytes gradually increased up to 9 per cent on the eleventh day of the experiment, and the whole cycle of reticulocyte formation lasted until the seventeenth day of the experiment. The actual number of reticulocytes present per cubic millimeter of blood at the peak of their production was 450,000 as compared to the 153,600 during the control period. The per cent of hemoglobin and the total number of erythrocytes reached normal levels nine days, sometimes ten, after hemorrhage on the one hundred and forty-fifth or fifteenth day of the experiment, and fluctuated near that level, steadily working upward, however, until the end of the experiment.

TABLE I

YOUNG RABBIT NO. 1 (MALE)

Aged 6-7 Mo. Weight 2,250 Gm. Bled 40 c.c. or One-Third of Blood

DATE OF EXPERIMENT	DATE	R.B.C. MILLION PER CU. MM. OF BLOOD	PER CENT HEMOGLOBIN	TOTAL CELLS COUNTED	TOTAL RETICULOCYTES	PER CENT OF RETICULOCYTES
	1932					
1	11/14	6.4	----	1,199	30	2.5
2	11/15	6.5	----	1,302	29	2.2
3	11/16	6.4	----	1,230	32	2.5
4	11/17	6.3	93.0	1,346	36	2.6
5	11/18	6.4	93.5	1,340	29	2.2
6	11/19	6.5	94.4	1,056	30	2.7
7	11/20	---	Bled 30 c.c. of blood		--	--
8	11/21	4.1	56.0	1,037	58	5.1
9	11/22	4.6	62.0	1,209	80	6.6
10	11/23	4.8	69.0	1,132	87	7.6
11	11/24	5.3	76.0	1,063	96	(9.0)
12	11/25	5.6	84.0	1,075	75	7.0
13	11/26	5.9	88.0	1,052	59	5.6
14	11/27	5.9	87.0	1,122	50	4.5
15	11/28	6.4	93.0	1,067	28	2.6
16	11/29	6.6	99.0	1,101	32	2.9
17	11/30	6.0	99.0	1,065	20	1.9
18	12/ 1	5.8	95.0	1,195	31	2.6
19	12/ 2	6.3	93.0	1,299	39	3.0
20	12/ 3	6.5	99.0	1,088	37	(3.2)
21	12/ 4	6.7	101.0	1,237	29	2.3
22	12/ 5	6.7	----	895	14	1.5
23	12/ 6	6.5	100.0	1,107	15	1.3
24	12/ 7	7.3	105.0	985	19	1.9
25	12/ 8	7.3	96.5	1,486	24	1.7
26	12/ 9	6.2	96.0	1,055	20	1.9
27	12/10	6.5	100.5	1,226	25	2.0
28	12/11	6.4	92.0	1,221	28	(2.3)
29	12/12	6.7	94.0	1,027	21	2.0

The rate of reticulocyte production remained higher than during the control period until the number of erythrocytes and the per cent of hemoglobin were almost normal on the fourteenth or fifteenth day of the experiment. On the sixteenth day the erythrocyte number was actually above the control period. The reticulocyte curve continued to fall for two or three more days, fifteenth to seventeenth day. On the eighteenth and nineteenth days there was a rather sudden drop, which amounted to almost a million per cubic millimeter of blood in the number of erythrocytes. The hemoglobin curve followed the erythrocyte

curve, more or less, except that there seemed to be a slight tendency for the hemoglobin curve to lag behind the erythrocyte curve toward the end of the experiment.

The rapid fall in number of erythrocytes on the seventeenth and eighteenth days was equal to a hemorrhage and can well be called an intravascular hemorrhage. It was sufficiently severe to stimulate another wave of reticulocyte production. This peak of reticulocyte production was on the twentieth day. As a result, the erythrocyte number returned to normal and even went quite above normal, reaching a new peak on the twenty-fourth and twenty-fifth days. At the same time the reticulocytes fell below the normal production. The third slight peak of reticulocyte production on the twenty-eighth day can scarcely be regarded as a response to the drop in erythrocytes on the twenty-sixth day, since the total does not go below normal. Because the total red corpuscle count ran above normal, the per cent of reticulocytes was below normal.

TABLE II

OLD RABBIT NO. 2 (MALE)

Aged 5½ Yr. to 6 Yr. Old. Weight 3,300 Gm. Bled 40 c.c. or One-Fourth of Blood

DATE OF EXPERIMENT	DATE	R.B.C. MILLION PER CU. MM. OF BLOOD	TOTAL CELLS COUNTED	TOTAL RETICULOCYTES	PER CENT OF RETICULOCYTES
	1932				
1	10/ 6	---	993	16	1.71
2	10/ 7	6.3	1,054	18	1.70
3	10/ 8	6.2	1,059	16	1.5
4	10/ 9	6.0	1,137	19	1.7
5	10/10	5.9	977	13	1.3+
6	10/11	5.8	1,510	29	1.9
7	10/12	5.9	835	10	1.2
8	10/13	---	1,241	17	1.3+
9	10/14	---	1,088	21	1.9
10	10/15	---	Bled 35-40 c.c. of blood		---
11	10/16	---	----	--	---
12	10/17	4.5	1,112	38	3.4
13	10/18	4.4	1,053	64	(6.0)
14	10/19	4.7	1,008	42	4.1
15	10/20	4.6	1,079	40	3.8
16	10/21	4.6	1,074	35	3.2
17	10/22	3.7	949	21	2.2
18	10/23	3.4	1,123	23	2.0
19	10/24	4.0	1,339	51	3.8
20	10/25	4.2	1,110	52	(4.6)
21	10/26	3.9	971 Abnormal erythrocytes appear	37	3.8
22	10/27	4.4	1,083	32	3.0
23	10/28	3.8	1,198	37	3.0
24	10/29	3.8	1,046	43	4.1
25	10/30	---	Died	--	---

The period of time between the greatest production of reticulocytes on the eleventh day and the fall in the number of erythrocytes about the eighteenth day must be regarded as the average length of life of the red corpuscle. This period is approximately seven days. It is also quite possible that the time between the second peak of reticulocyte production at the nineteenth or twentieth days, and the fall in erythrocyte number on the twenty-sixth day, would represent the same thing. Here, too, the time is approximately seven days.

Old Rabbit.—An experiment similar to the above was performed on an old rabbit (5½ to 6 years), the details of which are presented in Fig. 2. The experiment covered a period of twenty-four days, at which time the animal died. This rabbit, although old, was in good condition until on or near the twenty-first day, three days before death, the rabbit showed signs of weakness and diarrhea, probably due to too large an amount of green food during the weakened period subsequent to hemorrhage. The reticulocytes stained more deeply at this time, and can be considered polychromatophiles. The important part of the experiment was completed before these symptoms appeared.

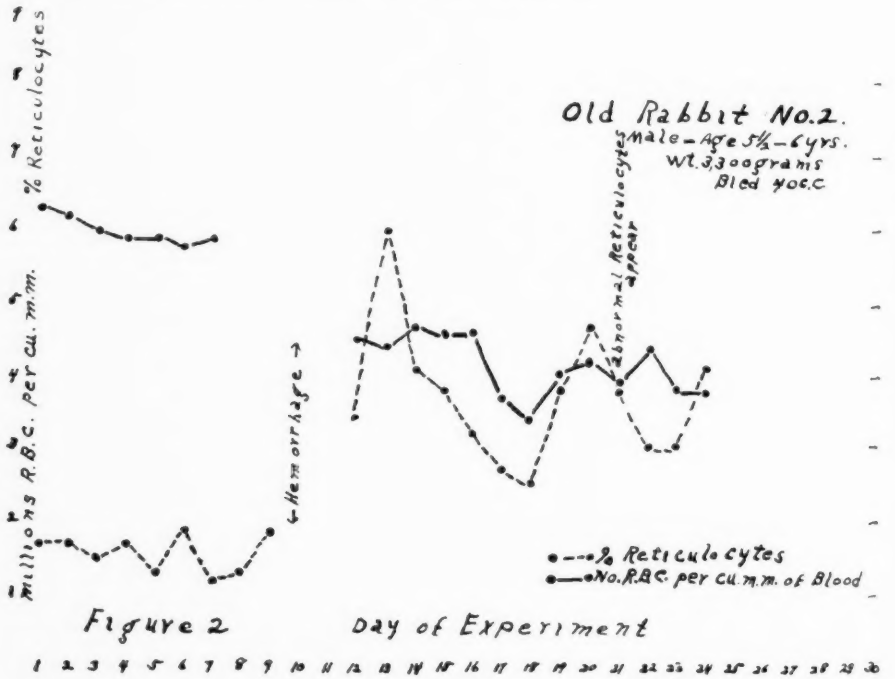

% Reticulocytes
Millions R.B.C. per cu. m.m.
Hemorrhage
Old Rabbit No.2.
Male-Age 5½-6yrs.
Wt. 3300grams
Bled 40c.c
Abnormal Reticulocytes appear
●--● % Reticulocytes
●—● No. R.B.C. per cu.m.m. of Blood
Day of Experiment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 2

Fig. 2.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of 40 c.c. of blood, approximately one-fourth of its blood. These were taken on the day before the day of the hemorrhage and again after seventy-two hours. Two days after the hemorrhage two peaks of reticulocyte formation occurred on the 13th and 20th days of the experiment. The total red corpuscle count was lowest on the 18th day of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 13th day and the 18th day. The average length of life of the red corpuscles was 5 days.

The hemoglobin was not tested in this experiment, since it was not deemed necessary. The control period lasted nine days. The average number of erythrocytes was six million, and the percentage of reticulocytes was 1.6 per cent. Tests were not made on the day of the hemorrhage, but they were made forty-eight hours later as well as on every subsequent day thereafter. On the twelfth day of the experiment, forty-eight hours after hemorrhage, there was a drop in erythrocytes to 4.5 millions and a rise in reticulocytes both in percentage and in actual number per cubic millimeter of blood.

The greatest percentage of reticulocytes occurred on the thirteenth day and the greatest destruction of erythrocytes occurred on the eighteenth day. The length of time between the peak of reticulocytes and the fall in the total number

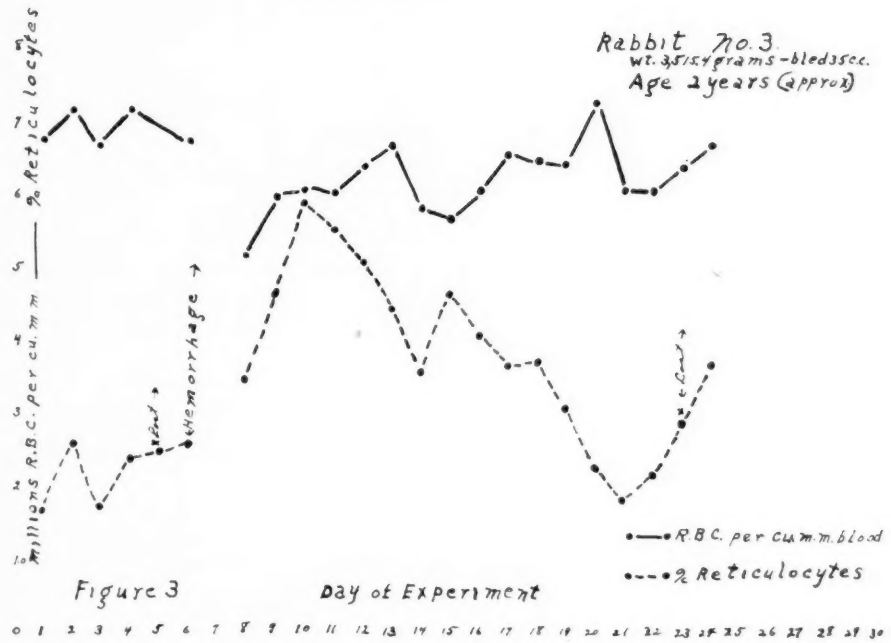

Rabbit No. 3
wt. 3515 grams - bled 35 cc.
Age 2 years (approx)
Millions R.B.C. per cu.m.m. — % Reticulocytes
Hemorrhage
R.B.C. per cu.m.m. blood
% Reticulocytes
Day of Experiment
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 3

Fig. 3.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Two peaks of reticulocyte formation occurred on the 10th and 16th days of the experiment. The total red corpuscle count was lowest on the 15th and 22nd days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 10th and the 15th days and between the 16th and 22nd days, respectively. The average length of life of the red corpuscles was estimated as 5.5 days.

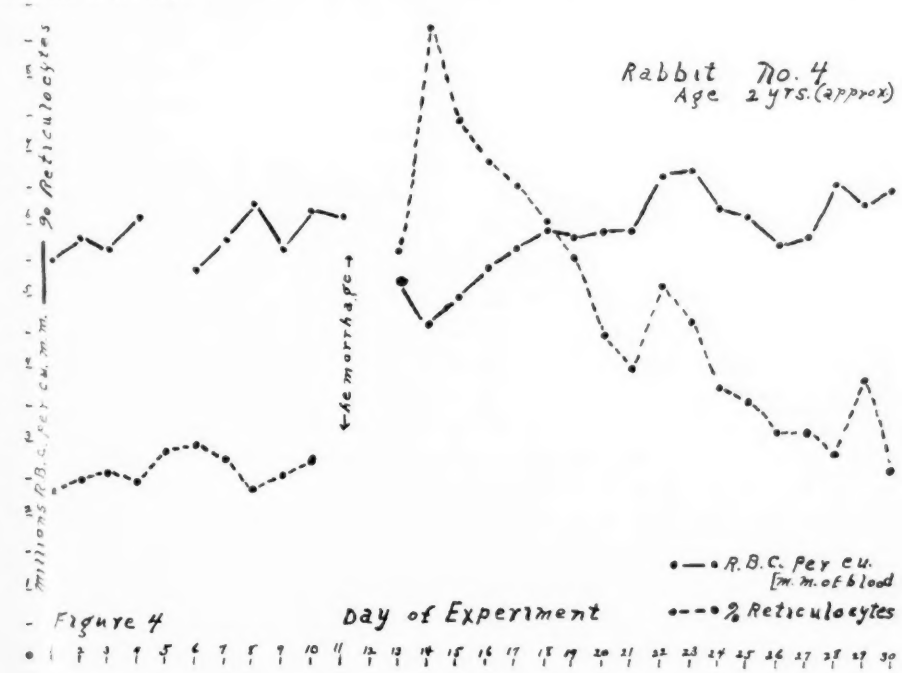


Figure 4

Fig. 4.—The per cent of reticulocytes and the total red corpuscle estimate were taken before and after a hemorrhage of approximately one-fourth of its blood. These were taken on the day of the hemorrhage and again after forty-eight hours. Two peaks of reticulocyte formation occurred on the 14th and 22nd days of the experiment. The total red corpuscle count was lowest on the 19th or 20th and on the 26th or 27th days of the experiment. The average length of life of the red corpuscles can be estimated by counting the days between the 14th and 19th or 20th days and between the 22nd and 26th or 27th days. The average length of life of the red corpuscles was estimated as 5.5 days.

of corpuscles per cubic millimeter of blood was five days. This five-day period must be considered the average length of life of the red corpuscles of an old rabbit.

It will be noticed that the number of erythrocytes never returned to the normal level after hemorrhage.

Middle-Aged Rabbits.—The tables showing data for these two rabbits were omitted to save space, but the data of these tables were used in constructing the two graphs, Figs. 3 and 4, as well as Table III, where a comparison of all four rabbits was made. The average length of life of the corpuscles of each of these two rabbits was five to six days.

TABLE III

COMPARATIVE STUDY OF TOTAL BLOOD COUNT AND RETICULOCYTE PERCENTAGE OF FOUR RABBITS OF DIFFERENT AGES

RABBIT	R.B.C. PER CU. MM. OF BLOOD (CONTROL PERIOD)	RETICULOCYTE PERCENTAGE (CONTROL PERIOD)	AVERAGE LENGTH OF LIFE OF R.B.C.	R.B.C. MADE EACH DAY PER CU. MM. OF BLOOD	NO. OF RETICULOCYTES ACTUALLY PRESENT AT ANY ONE TIME PER CU. MM. OF BLOOD (CONTROL)	DURATION OF RETICULOCYTE STAGE
Young rabbit No. 1 Aged 6-7 mo.	6,400,000	2.4	6-7 days Avg. 6.5 days	984,615	153,600	3 hr. 45 min.
Middle-aged rabbit No. 3 Aged slightly less than 2 yr. (approx.)	6,850,000	2.39	5-6 days Avg. 5.5 days	1,245,454	163,715	3 hr. 10 min.
Middle-aged rabbit No. 4 Aged 2 yr. (approx.)	5,880,000	2.63	5-6 days Avg. 5.5 days	1,069,000	137,000	3 hr. 4 min.
Old rabbit No. 2 Aged 5½-6 yr.	6,000,000	1.6	5 days	1,200,000	96,000	1 hr. 55 min.

DISCUSSION—COMPARISON

The blood pictures of the young rabbit, Fig. 1, and the old rabbit, Fig. 2, were very similar during their respective control periods. The erythrocyte count of the young animal averaged 6.4 million, and the count of the old animal averaged 6 million. The reticulocyte percentage of the young rabbit averaged 2.4 and that of the old rabbit was 1.6. An amount of blood calculated to be approximately one-fourth to one-third of the total blood volume was removed from each animal at the end of the control period. Forty-eight hours were allowed to elapse before the daily tests were begun again.

The initial reticulocyte response of the young rabbit was greater and lasted over a longer period of time than that of the old rabbit, while the second peak of reticulocytes was higher in the old rabbit than in the young one. The time between the reticulocyte peaks for the young and old rabbits was nine and seven days, respectively. The explanation of this difference may be that the young rabbit produces enough reticulocytes during the first response to

bring the number of erythrocytes back to normal level, while the hemopoietic response in the case of the old animal was never sufficient to restore the original number of erythrocytes. Furthermore, more erythrocytes died at one time in the old animal and precipitated a higher second reticulocyte peak proportionately than in the case of the young animal.

On the ninth day after hemorrhage the young rabbit had its erythrocytes restored to a low normal, while, on the other hand, the old rabbit never returned to normal; in fact, it scarcely gained in number of erythrocytes at all.

Although the reticulocyte production was not complete enough and the cycle was not long enough before the subsequent intravascular hemorrhage occurred, due to a short length of life of the corpuscles, the conclusion reached was that the animal's red corpuscles decreased in number with each subsequent intravascular hemorrhage.

Seven days after the peak of reticulocytes in the young rabbit, there was a fall in total number of erythrocytes of approximately a million. In like manner five days after the peak of reticulocytes in the old rabbit, there was a fall in the total number of reticulocytes of a million or more. It cannot be doubted that the fall of a million in total number of erythrocytes in both animals on the eighteenth day of each experiment was due to an intravascular hemorrhage, a loss of erythrocytes born at the previous peak of reticulocyte formation. A second peak of reticulocytes, followed by an intravascular hemorrhage, was shown in the young rabbit. The period was between the nineteenth or twentieth days and the twenty-sixth day of the experiment.

Since the period of seven days in the young animal and the period of five days in the old animal must represent the average length of life of the red corpuscles in the two animals, the conclusion would be that the erythrocyte lives longer in the blood stream of a young animal than it does in the blood stream of an old one. This conclusion is further borne out in the results from two middle-aged (2 years old) rabbits in which the average length of life of the corpuscles was 5.5 days (see Table III).

The time between the hemorrhage and the first peak of reticulocyte response, five days and four days, respectively, in the young and old rabbits, was greater than the time between the intravascular hemorrhage and the succeeding reticulocyte response, two days each in young and old. The explanation, no doubt, is that the intravascular hemorrhage did not involve loss of volume; therefore, the time should be correspondingly decreased. Robertson and Boch (1919)[14, 15] have shown that it is the reduced hemoglobin per unit of blood due to dilution of the blood after hemorrhage that is the stimulating factor in reticulocyte formation.

Knowing the total number of erythrocytes per cubic millimeter of blood and the percentage of reticulocytes, the actual number of reticulocytes per cubic millimeter of blood can be determined: 6,000,000 × 1.6 per cent = 96,000 reticulocytes (see Table III, old rabbit).

Knowing the length of life of the red corpuscles, the number of corpuscles made each day may be determined: 6,000,000 ÷ 5 = 1,200,000 (see Table III, old rabbit).

Knowing the foregoing, the reticulocyte period can be determined: 1,200,000 ÷ 96,000 = 1/12.5 of a day, or one hour and fifty-five minutes (see Table III, old rabbit).

By similar calculation the reticulocyte stage in a young animal was found to last three hours and forty-five minutes. The comparative data are set out in Table III.

Calculations made for the two other rabbits shown in Table III are also tabulated. In practically every respect the blood pictures of the two, 2-year-old rabbits show conditions midway between the young and old rabbits.

The ability of the rabbit to recover from hemorrhage is due to a combination of the length of life of the corpuscle and of the number of corpuscles thrust into the blood stream in twenty-four hours. In the case of the young rabbit No. 1 the recovery was rapid because the length of life of the corpuscle is long and the number of reticulocyte percentage is high. The old rabbit No. 2 showed the other extreme, the reticulocyte percentage being low and the average length of life of the corpuscle short. It was unable to recover. Rabbits Nos. 3 and 4 recovered easily because the reticulocyte percentage was high, although the length of life of the corpuscles was somewhat shorter than in the case of rabbit No. 1.

Furthermore, the actual number of reticulocytes produced per cubic millimeter of blood every twenty-four hours was actually greater during the control period than that of the young rabbit; e.g., the young rabbit produced 984,615 reticulocytes per cubic millimeter of blood as compared to 1,200,000 produced by the old rabbit per cubic millimeter of blood.

CONCLUSIONS AND SUMMARY

1. After hemorrhage the reticulocyte cycles follow one another at regular intervals of a few days. The second reticulocyte cycle and each succeeding one is preceded by an intravascular hemorrhage in which corpuscles are destroyed but the volume of blood remains constant.

2. The longest period noted between reticulocyte cycles was nine days in a young rabbit, and the shortest period was seven days in an old rabbit.

3. The average length of life of the red corpuscle is equal to the period between the peak of a reticulocyte cycle and the end of the succeeding intravascular hemorrhage.

4. The average length of life of the red corpuscle of a rabbit varies inversely with age—the younger the rabbit, the longer the corpuscles live.

5. The average length of life of the red corpuscle varies from six to seven days in a young rabbit to five days in an old rabbit.

6. The extreme length of life of a red corpuscle may be much longer than the average life because many may be destroyed in "infancy" or in "early adult life."

7. The number of red corpuscles liberated into the blood stream each twenty-four hours by a young rabbit was 984,615 per cubic millimeter of blood, and by an old rabbit, 1,200,000 per cubic millimeter of blood.

8. The number of reticulocytes present per cubic millimeter of blood was 153,600 for a young rabbit, 96,000 for an old rabbit, and 1,245,451 and 1,069,000 for two middle-aged rabbits.

9. The duration of the reticulocyte stage was approximately four hours in a young rabbit and two hours in an old rabbit. The reticulocyte stage in the two other rabbits, aged 2 years, was about midway between that of the old and young animals; namely, 3 hours 10 minutes, and 3 hours 4 minutes, respectively.

10. An accurate picture of the activity of the red bone marrow can be obtained if the number of red corpuscles per cubic millimeter of blood, the average length of life of the red corpuscles, the per cent of reticulocytes present in the blood, and the duration of the reticulocyte stage of the red corpuscle are known.

I take this opportunity to thank Dr. William Moenkhaus and Dr. Paul Harmon, of the Physiology Department of Indiana Medical School, for help and encouragement.

REFERENCES

1. Ashby, Winifred: The Determination of the Length of Life of the Transfused Blood Corpuscles in Man, J. Exper. Med. **29**: 267, 1919.
2. Ashby, Winifred: The Periodicity in Eliminative Activity Shown by the Organism, J. Exper. Med. **34**: 127, 1921.
3. Ibid.: **34**: 147, 1921.
4. Cunningham, T. D.: A Method for the Permanent Staining of Reticulated Red Cells, Arch. Int. Med. **26**: 405, 1920.
5. Eaton, Paul, and Damren, F. L.: A Method for Determining the Life Duration of the Erythrocyte, South. M. J. **23**: 311, 1930.
6. Ibid.: **23**: 395, 1930.
7. Escobar, R. A., and Baldwin, F. M.: The Longevity of the Erythrocyte, Am. J. Physiol. **107**: 249, 1934.
8. Harne, O. G., Lutz, John F., and Davis, Carl L.: Induced Reticulocytosis in the Rat and Its Relation to the Life Duration of the Red Blood Cell, J. Lab. & Clin. Med. **25**: 333, 1940.
9. Hunter, William: The Duration of Life of Red Blood Corpuscles After Transfusion, in Its Transfusion in Man, Brit. M. J. **1**: 192, 1887.
10. Hunter, William: Ibid., Proc. Roy Soc. Edinburgh **13**: 849, 1884-86.
11. Key, J. Albert: Studies on Erythrocytes, With Special Reference to Reticulum, Polychromatophilia, and Mitochondria, Arch. Int. Med. **28**: 511, 1921.
12. King, J. T.: Secretin Not a Hematopoietic Stimulant, University of Minnesota Contributions From the Department of Physiology, Reprint from Arch. Int. Med. **42**: 762, 1928.
13. Rous, Peyton: Destruction of Red Blood Cells, Physiol. Rev. **3**: 75, 1923.
14. Robertson, O., and Boch, A.: Blood Volume in Wounded Soldiers, J. Exper. Med. **29**: 133, 1919.
15. Ibid.: p. 155.
16. Spohr, C. L.: Reticulocytes, Their Identification and Significance, J. Lab. & Clin. Med. **15**: 474, 1930.
17. Wean, J. T., and Warren, S.: The Length of Life of Transfused Erythrocytes in Patients With Secondary and Primary Anemia, Arch. Int. Med. **29**: 527, 1922.
18. Whipple, G. H.: The Hemoglobin of Striated Muscle, Am. J. Physiol. **76**: 693, 1926.

THE EFFECT OF LOWERED TEMPERATURES UPON THE GROWTH OF THE FIBROBLAST IN VITRO: ITS APPLICATION TO WOUND HEALING*

MACHTELD E. SANO, M.D., AND LAWRENCE W. SMITH, M.D.
PHILADELPHIA, PA.

IN THE course of tissue culture studies relating to the growth of embryonal and cancer cells under the influence of varying temperatures,† the behavior of the fibroblast has been so outstanding that it merits separate consideration. The vital importance of the role of this cell in wound healing and repair processes in general makes our understanding of its unusual resistance to cold and its adaptability to unfavorable environmental circumstances of especial significance.

At body temperature (37° C.) and under ideal culture conditions, in respect to material and media, the fibroblast multiplies rapidly and produces large, elongated, healthy appearing cells, with obvious coarse fibroglia fibers. It is not necessary to go into great detail regarding this aspect of the problem, as the behavior of the fibroblast under such circumstances has been thoroughly investigated by many competent observers.[3, 5-7, 10] Like these other investigators we have found that the rate of such normal growth is not maintained for any considerable period in vitro unless the cultures are transplanted at frequent intervals. Otherwise the cells revert to what might be termed a "resting" or resistant phase, not unlike the encystment stage of certain parasites, only to grow again at a normal rate after a brief lag period when transferred to fresh media.

The resistance to lowered temperatures of the fibroblast is very marked. Initial congelation at 0° C. for as long as sixty hours does not significantly alter the growth capacity of these cells when the cultures are subsequently placed in the incubator at 37° C., other than for the usual lag noted previously in the tumor cell cultures. Nor is any inherent difference in the growth noted in parallel cultures with the temperature lowered gradually from an initial 37° C., as compared with those started off at the corresponding lower levels.

At 30° C. obvious differences in the appearance of the fibroblasts from those grown at 37° C. are seen. The cells are smaller both in respect to the size of the nucleus and the amount of cytoplasm. This decrease in size is especially prominent in respect to the length of the cell, the whole cell appearing more compact and sturdy, with ovoid rather than spindled nuclei.

*From the Department of Pathology, Temple University Hospital and School of Medicine, Philadelphia.

Aided by grant No. 580 of the Committee on Scientific Research of the American Medical Association, and a grant from the Clinical Research Foundation, Philadelphia.

Received for publication, December 20, 1940.

†In press. Cancer Research, January, 1942.

At 25° to 26° C. the fibroblasts are scarcely a third the size of those grown at 37° C. and show only little intercellular substance. Instead of migrating freely and extending in long fingerlike projections from the mother colony, they are arranged in almost solid radiating phalanx fashion, several cells deep, around the original explant. The cytoplasmic and nuclear detail is well defined in both unstained and vitally stained specimens.

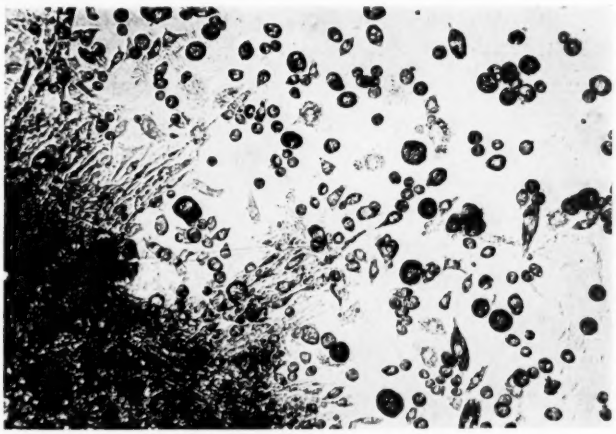


Fig. 1.—Normal mouse lymph node at 37° C. Approximate magnification, ×150.

At 20° C. the rate of growth is still further retarded. Six or seven days elapse before the first evidence of new cell formation can be made out. At this temperature level the cells are likewise still further diminished in size, scarcely a fifth that of those grown at 37° C. They are very short and stubby in appearance, almost round or polyhedral rather than ovoid or spindled. As they slowly increase in numbers they occur in small sheets at the edge of the original inoculum, but it takes several weeks for this to occur.

At the 24° to 26° C. temperature levels the cells are far more resistant to environmental factors. This may be accounted for in several ways. Owing to their more compact arrangement they are less readily damaged physically by washing and changing their media. Owing to their slower rate of growth they survive in the original medium for a much longer period of time than the larger more rapidly growing fibroblasts at 37° C. This is undoubtedly due in part to their lower nutritional requirements, but also to the reduction in the metabolism of the cells, with a corresponding reduction in the concentration of the products of metabolism in the medium. This latter feature has been stressed by many writers, as well as ourselves in other communications, as being one of the very important factors which interfere with tumor cell culture. As part of this slowed metabolism the usual regressive degenerative changes of the cytoplasm of cells grown "in vitro" are obviously retarded, which in turn fits in with the previous comments in helping to explain the increased resistance of these cells to lowered temperatures.

We believe that these observations are well established and might be expressed almost mathematically because of their constancy in the case of literally

hundreds of such tissue explants. There is a direct proportional relationship not only between the temperature and the rate of cell growth, but also in respect to temperature and the size of the cell, its degree of differentiation, its metabolic activity, and its resistance to environmental circumstance. On the basis of these studies a temperature range of 25° to 30° C. would seem to be optimal in respect to wound healing. Under such circumstances one logically might expect that the healing process would be somewhat slower than at body temperature, but that the amount of collagen production, with its subsequent ugly contraction and scar formation, would be reduced appreciably.

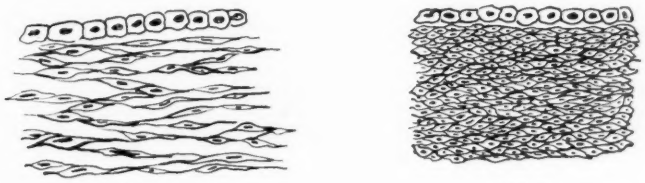
That this optimal reaction actually does occur clinically was shown by Smith and Fay[12] incidentally in their early studies concerning the effect of hypothermy on cancer cell growth. In one case, especially, of extensive ulcerative breast carcinoma treated by continuous local hypothermy for nearly six months, not only did the tumor disappear, but the ulcerated surface healed over completely with soft scar tissue having the consistency of normal skin. Histologic study of biopsy material from this and numerous other cases confirmed the clinical impression. The tissues showed a connective tissue relatively rich in nuclei and with a minimum of dense collagen formation.

Fig. 2.—Normal mouse lymph node at 30° C. Approximate magnification, ×150.

The recent work of Allen[1] and his associates in the healing of diabetic and traumatic amputation stumps under the influence of cold is another illustration of the clinical application of this principle. In almost every instance healing by first intention resulted, and with a minimum of scar production. The repair process, as might be expected, was quite regularly delayed, requiring three to four weeks instead of the usual ten days to two weeks. On the other hand, the wounds did not break down, and the stumps presented a much more satisfactory end result for subsequent artificial limb appliances. As part of the mechanism involved in the use of hypothermy for such wound repair, it should be noted that the external temperatures employed (5° to 10° C.) are bacteriostatic, and thus the danger of secondary infection is almost eliminated. It should be further mentioned that actual freezing must be avoided, and that the penetra-

tion of the cold into the deeper tissues from local external application drops off with almost algebraic progression, so that the desired levels of 20° to 25° C. are actually present in the deeper tissues of the wound where the connective tissue repair process is going on.

On the basis of such clinical observations and the confirmatory laboratory evidence of the behavior of the fibroblast at these lower temperatures, the more widespread use of hypothermy in wound healing would seem to be warranted. Its application in plastic surgery, of the cosmetic type particularly, comes to mind immediately. The possible prevention of keloid formation is another field which might profitably be explored. Its place in clearing up old osteomyelitis lesions might likewise be investigated.



37° C. 30° C.

A. *B.*

Fig. 3.—Schematic representation of fibroblastic proliferation at *A*, 37° C. and *B*, 30° C. Note the smaller size, compactness, and diminished intercellular products at the lower temperature.

It is of interest that these various tissue culture studies and clinical observations of ours are indirectly confirmatory of the fundamental work of Burrows[4] on wound healing, although the approach to the problem is from a wholly different angle. Burrows noted that the growth of the fibroblast in his experimental wounds at body temperature was most profuse in the central areas which were less well vascularized than in the periphery of the lesion. He advanced the hypothesis that the fibroblasts grow better when they are closely packed together so that certain metabolic products of oxidation accumulate in optimal amounts, through their inability to be absorbed and carried away by an inadequate blood supply. By corollary he comments that if the wound is washed too frequently, the rate of growth of the fibroblasts is likewise reduced, and healing is thereby delayed and less satisfactory.

In our own studies the reduction of temperature to 25° or 30° C. gives us similar optimal conditions for satisfactory wound healing. The cells are growing vigorously; they are closely packed and the circulation is slowed. Thus the products of metabolism are in close contact with the cells, and remain so, through the inability of the slowed blood stream to carry them away. In addition these temperatures are bacteriostatic to a very considerable degree and aid materially in checking any infection which might be present (Fig. 3).

Finally, there is a definite relationship between epithelialization and fibroblastic proliferation in a wound. When the surface becomes covered with epithelium, the stimulus to fibroblastic proliferation is lost. If this epithelialization is accomplished too rapidly, the underlying granulation tissue tends to undergo regressive changes, the collagen contracts, and we have a depressed secondarily

contracted scar. If, on the other hand, the repair process is slower, the connective tissue is adequate in amount, compactly arranged, with relatively little collagen, and the final scar is minimal with little or no retraction. From our observations all of these prerequisites for good wound healing seem to be realized by temperatures between 25° and 30° C.

SUMMARY

The behavior of the fibroblast in tissue culture at varying temperatures ranging from 0° to 37° C. is recorded. An optimal level of around 25° C. is suggested as of practical value in wound healing.

Clinical observations of the use of hypothermy in various fields, including a review of some of the more recent literature, are presented.

A plea for the wider use of local hypothermy in various clinical problems relating to wound healing is made.

REFERENCES

1. Allen, F. M.: Reduced Temperatures in Surgery, Am. J. Surg. **52**: 225, 1941.
2. Bloom, W.: Cellular Differentiation and Tissue Culture, Physiol. Rev. **17**: 589, 1937.
3. Boitsell, G. A.: The Origin and Structure of a Fibrous Tissue Formed in Wound Healing, J. Exper. Med. **23**: 739, 1916.
4. Burrows, M. T.: Studies in Wound Healing, J. M. Research **44**: 615, 1924.
5. Carrel, A.: Contributions to the Study of the Mechanism of the Growth of Connective Tissue, J. Exper. Med. **18**: 287, 1931.
6. Erdmann, R.: Einige grundlegende Ergebnisse der Gewebezüchtung aus den Jahren 1914-1920, Ergebn. d. Anat. u. Entwcklngsgesch. **23**: 419, 1920.
7. Fisher, A.: Factors of a Growth Regulating Nature in Tissue Cells, Am. J. M. Sc. **173**: 562, 1927.
8. Fukuda, E., Tuyama, M., and Yasurumi, S.: "Critical Reaction" of Fibroblasts, Okajinas Folia anat. japon. **19**: 127, 1940.
9. Krull, G.: Process of Healing Fractures Studied in Tissue Cultures, Arch. f. orthop. u. Unfall-chir. **37**: 131, 1936.
10. Lewis, W. H.: Cultural and Cytological Characteristics of Normal and Malignant Cells in Vitro, Arch. f. exper. Zellforsch. **21**: 316, 1938-1939.
11. Ludford, R. J.: Interaction in Vitro of Fibroblasts and Sarcoma Cells With Leukocytes and Macrophages, Brit. M. J. **1**: 201, 1940.
12. Smith, L. W., and Fay, T.: Observations on Human Beings With Cancer Maintained at Reduced Temperatures of 75°-90° F., Am. J. Clin. Path. **10**: 1, 1940.
13. von Möllendorff, W.: Mitosis: Phase Division in Cultures of Fibrocytes and Their Changes Following Addition of Various Substances, Ztschr. f. Zellforsch. u. mikr. Anat. **27**: 301, 1937.

AN UNUSUAL P-WAVE IN CHEST LEAD CF_2 FOLLOWING SPONTANEOUS PNEUMOTHORAX*

S. D. Burton, M.D., and J. S. Mehlman, M.D.
Chicago, Ill.

ALTHOUGH it is well recognized now that changes in the position of the heart will alter the electrocardiogram, little work has been done to illustrate these changes in the chest leads. Recently we have had the occasion to follow the changes in the electrocardiogram caused by a spontaneous pneumothorax which occurred in a patient who had an abnormal electrocardiogram due to congenital heart disease. The electrocardiographic changes were correlated with changes in position of the heart as the pneumothorax appeared and then resolved. A control record was available before the pneumothorax developed, and serial electrocardiograms were taken during the course of the patient's illness.

A striking alteration in the P-wave of chest Lead CF_2 was especially noted. The changes in this P-wave were similar to, but more marked, than those reported by Gertz[1] from this department. This record serves to emphasize again that such P-wave changes are suggestive of a position change of the heart.

CASE REPORT

The patient was a 19-year-old white male, who entered Michael Reese Hospital on the service of Dr. W. A. Brams on Nov. 12, 1939, complaining of cough and pain in the chest of one day's duration. He was known to have had heart disease since birth, and his activities had always been restricted. He had been followed in the out-patient clinic.

On the morning of admission he was awakened by pain in his right chest and a nonproductive cough. On two occasions following a paroxysm of coughing, he became dyspneic and perspired profusely. There was no precordial pain or hemoptysis.

His past medical history revealed the presence of cyanosis since birth, pneumonia at the age of 5, measles, and chickenpox. One brother had rheumatic heart disease. Three other siblings, his mother and father, were all alive and well. He had had a series of electrocardiograms taken before this episode, one of which is shown in Fig. 1*A*.

On admission he had a temperature of 99° F., a pulse rate of 120, and a respiratory rate of 28 per minute. The blood pressure was systolic 96 and

*From the Cardiovascular Department and the Department of Medicine, Michael Reese Hospital, Chicago.
Aided by the A. D. Nast Fund for Cardiac Research.
Received for publication, December 23, 1940.

diastolic 70. The patient was underdeveloped and thin. His skin was cyanotic. He was slightly dyspneic even with the back rest elevated at a 60 degree angle. There were no petechiae visible. The right hemithorax appeared larger than the left, and it moved less with respiration. Tactile fremitus was diminished, and the percussion note was hyperresonant over the entire right side of the chest. Breath sounds were absent, except for a small area of bronchial breathing beneath the clavicle. The pulmonary findings on the left side were normal. The apex beat was diffusely palpable in the fourth, fifth, and sixth left intercostal spaces as far laterally as the anterior axillary line. There was no cardiac dullness to the right of the sternum. The heart rhythm was regular, and the tones were loud. There was an accentuation of the first sound at the apex. No murmurs were audible at the time of this examination. The abdomen was negative. There was a moderate left thoracic kyphoscoliosis. Clubbing of the fingers and toes was marked. The diagnosis was congenital heart disease and right spontaneous pneumothorax.

An electrocardiogram was taken at this time (Fig. 1*B*). A teleoroentgenogram also taken on the day of admission showed a right pneumothorax with almost complete collapse of the upper and middle lobes against the mediastinum. The right lower lobe appeared 80 to 90 per cent collapsed. There was a slight upper dorsal scoliosis so that it was difficult to determine whether there was a shift of the heart or mediastinum to the left.

On Nov. 13, 1939, the venous pressure was 6.8 cm. of water, the circulation time (arm to tongue) by the calcium gluconate method was 15.5 seconds, and the vital capacity was 1,300 c.c. The intrapleural pressure on the right side varied between 0 and +4.0 cm. of water on quiet respiration. After 1,000 c.c. of air were aspirated from the right chest, the foregoing determinations were repeated. The vital capacity rose to 1,600 c.c., and the intrapleural pressure varied from –6.0 cm. to 0 cm. of water during respiration. Electrocardiograms were taken before and after aspiration of the air and are shown in Fig. 2, *A* and *B*. After aspiration Lead CF_2 was recorded with the patient in three different positions: (1) sitting up, (2) left lateral, and (3) right lateral (Fig. 3).

A chest film twelve hours after the aspiration of the air showed little change in the findings. Blood studies revealed an intense secondary polycythemia with a red blood cell count of 6,720,000, hemoglobin, 24.0 Gm. per 100 c.c. of blood, volume index of 1.1, color index 1.05 and a hematocrit of 71 per cent. Fluoroscopic examination on November 18 showed a persistence of the pneumothorax, with an almost total collapse of all three lobes of the right lung. The heart was slightly deviated to the left, with a rounded contour of its left border. The right ventricle was moderately enlarged in the right oblique. There was no left auricular or ventricular enlargement. The aortic knob was hardly visible.

The patient's course in the hospital was uneventful. Fluoroscopy on November 25, thirteen days after admission, showed 60 to 70 per cent collapse of the right lung still present. Another electrocardiogram was taken at this time (Fig. 1C). On December 4, twenty-two days after admission, 50 per cent collapse was observed on fluoroscopic examination. One week later almost all

physical signs of pneumothorax had disappeared. A loud systolic murmur at the apex transmitted toward the base was now audible. The remainder of the physical findings were unchanged. The possibility of a patent interventricular septum, moderate overriding of the aorta to the right ventricle, and narrowing of the infundibulum of the pulmonary artery were considered as the anatomic defects.

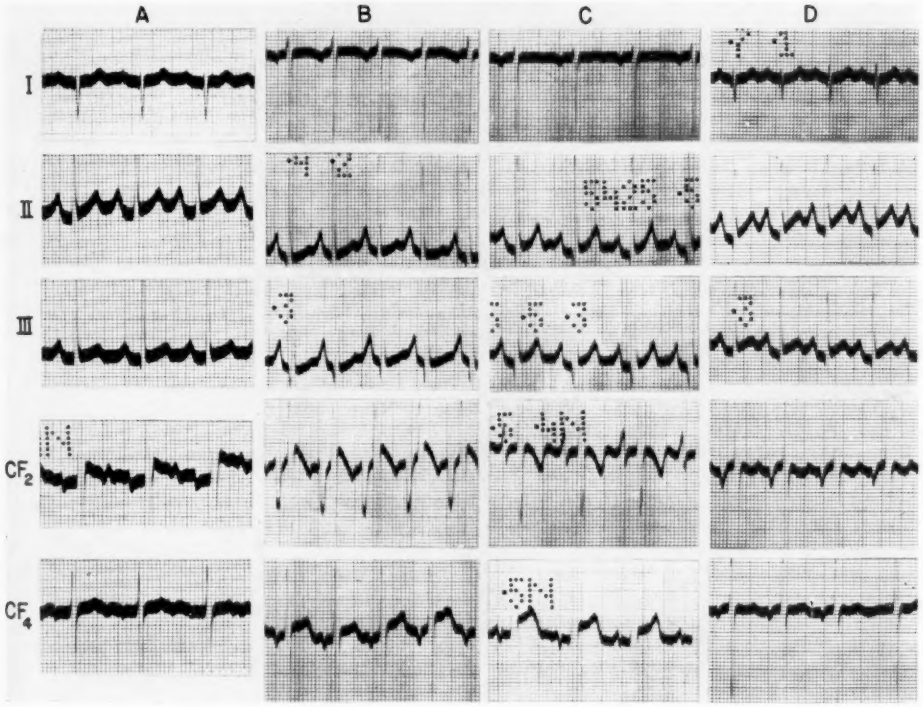


Fig. 1.—Effect of pneumothorax and its spontaneous resolution on the electrocardiogram. Segment A, control record taken on Jan. 9, 1939. Segment B, record taken on Nov. 13, 1939, after a spontaneous right pneumothorax. (Note the deeply inverted P-wave in Lead CF_2.) Segment C, record taken on Nov. 25, 1939, when the right lung was 60 to 70 per cent collapsed. Segment D, record taken on April 6, 1940, when all evidence of the pneumothorax had disappeared. See text for discussion.

The patient was discharged on December 12, thirty days after admission, much improved. Further observations were made in the out-patient department. A teleoroentgenogram was taken on Feb. 3, 1940, and showed no evidence of a pneumothorax. A final electrocardiogram was taken on April 6, 1940 (Fig. 1*D*). His course since discharge has been essentially similar to that before the pneumothorax.

DISCUSSION

Examination of Fig. 1 shows the effect of the pneumothorax upon the electrocardiogram. Record A, the control, is abnormal, showing a right ventricular preponderance and peaked P-waves in the limb leads. Following the pneumothorax (segment B), marked changes in the S-T segment and T-wave were produced, and there was an increase in the axis deviation to the right. The most striking changes occurred, however, in the P-waves, and especially the

P-wave in Lead CF_2; this deflection consisted of a deeply inverted wave 14 mm. in amplitude, preceded by a tiny preliminary upright phase. With partial resolution of the pneumothorax, segment C, the electrocardiogram tended to revert toward its previous appearance and P-wave changes occurred in all leads. The P-wave in CF_2 was still large, but it had become diphasic with the first phase up. The record, segment D, taken after all evidence of the pneumothorax had disappeared is fairly similar to the control record taken before the

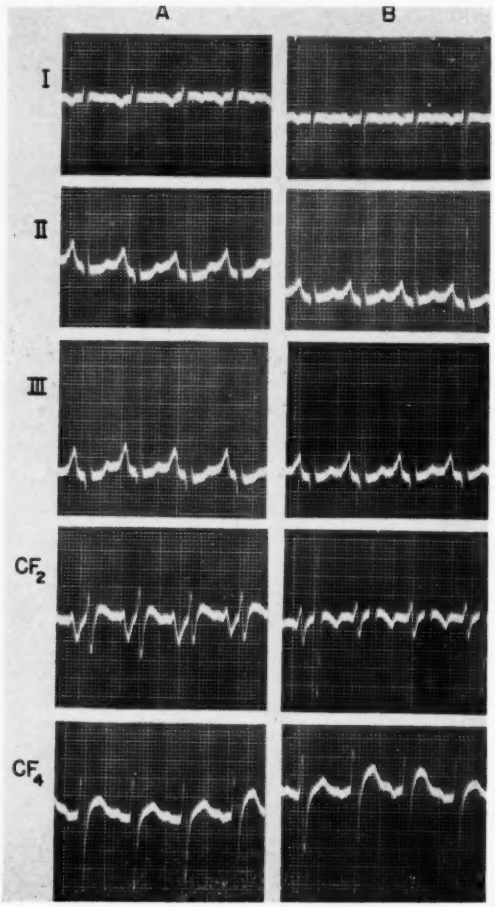


Fig. 2.—Effect of partial release of pneumothorax on the electrocardiogram. Segment A, control record (compare with segment B, Fig. 1). Segment B, record taken immediately after 1,000 c.c. of air had been withdrawn from the right pleural cavity. Note the decrease in the right axis deviation and the marked change in the P-wave in Lead CF_2.

pneumothorax developed. It is, therefore, proper to ascribe the electrocardiographic alterations in this case to the pneumothorax. This statement is further supported by comparing the similarity in the changes which occurred immediately following the withdrawal of 1,000 c.c. of air from the chest (Fig. 2) and the more gradual changes illustrated in Fig. 1 (*B*, *C*, and *D*) as the process resolved spontaneously.

The P-wave in Lead CF_2 in these figures, although larger in amplitude, is similar to the one described by Gertz.[1] It is the type of deflection obtained by

Wilson, Macleod, and Barker[2] and others when a unipolar lead with one electrode connected directly to the auricle is applied. However, the phase relations of the P-wave in this case and in that of the authors just mentioned are mirror images because the lead connections to the galvanometer were reversed.

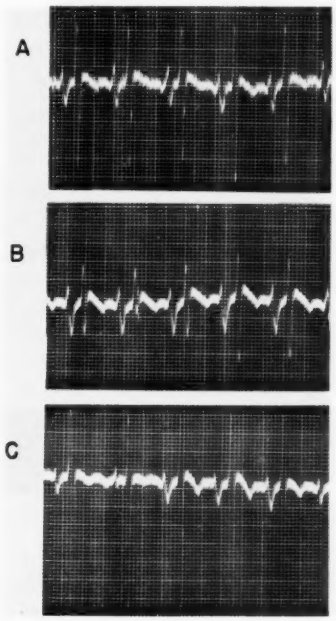


Fig. 3.—Records showing effect of postural changes in the electrocardiographic contour of the chest Lead CF_2 in this patient when the pneumothorax was present. Segment A, patient sitting upright. Segment B, patient in the left lateral recumbent position. Segment C, patient in the right lateral recumbent position. (Note the marked P-wave changes and the variations of the P-wave with respiration in segment C.)

The P-wave changes in this case must be ascribed to a displacement of the heart, such that the auricle comes to lie beneath the chest electrode. When the pneumothorax was at a maximum, viz., in Fig. 1*B* and Fig. 2*A*, the appearance of the P-wave suggests that the part of the auricle in which the pacemaker of the heart is located was lying close to the chest electrode. Such records, or rather their inverted images, because of the reversed electrode connections, have been obtained with unipolar leads in the neighborhood of the sinus node. In order for these P-wave changes to occur, it must be presupposed that the heart is pushed over laterally, ventrally, and caudally, and that it is also rotated in a counterclockwise direction when viewed from above. Counterclockwise rotation causes a deviation of the electrical axis to the right (Katz and Robinow[3]); this was observed in our case.

With resorption of the pneumothorax, the displacement of the heart was less marked. Hence the region of the sinus node became farther removed from the chest electrode, and at this stage a P-wave with more nearly equal phases was recorded (Figs. 1*C* and 2*B*).

As might be expected, the heart in a chest with a partial pneumothorax is more mobile than normal. This is shown by the changes in the contour of the electrocardiogram in Fig. 3 when the body posture was altered. In addition,

the respiratory variation in the P-wave contour was greatest when the patient was in the right lateral position with his heart supported from below by the collapsed lung and the pneumothorax (Fig. 3*C*). These shifts in the heart's position alter the relation of the heart to the good and poor electrical conductors in contact with its surface and lead to the variations in the electrical field. It has been shown that the conductors surrounding the heart vary in their ability to conduct the electric currents generated by the heart and that alterations in these contacts affect the electrocardiogram (Katz and Korey;[4] Lindner and Katz[5]).

The view presented by Gertz[1] is thus confirmed and amplified: namely, that peculiar large P-waves in the chest Lead CF_2 may be a sign of cardiac displacement in which the right auricle is brought into close proximity with the chest electrode.

SUMMARY

A case is presented showing an unusual and deeply inverted P-wave of 14 mm. in Lead CF_2. This P-wave was produced by cardiac displacement, resulting from a spontaneous pneumothorax which brought the right auricle into close apposition to the anterior chest wall. The contour of the P-wave was such as to suggest that the part of the auricle beneath the chest contained the pacemaker, the sinus node. As the pneumothorax was relieved, the large P-wave became diphasic, suggesting the apposition of other parts of the auricle to the anterior chest wall. Finally, as further resolution of the pneumothorax progressed, the P-wave returned to its smaller and more nearly normal appearance, which existed before the pneumothorax occurred. During the period of pneumothorax, the changes in the P-wave with change in body posture indicated greater than normal mobility of the heart.

We are grateful to Dr. L. N. Katz, at whose suggestion this study was undertaken, for his advice and guidance.

REFERENCES

1. Gertz, G. F.: An Unusual P Wave in Lead IV, Am. Heart J. **15**: 498, 1938.
2. Wilson, F. N., Macleod, A. G., and Barker, P. S.: The Distribution of Currents of Action and Injury Displayed by Heart Muscle and Other Excitable Tissue, Univ. Mich. Press, pp. 8-12 and Fig. 3, 1933.
3. Katz, L. N., and Robinow, M.: The Appearance of the E.C.G. in Relation to the Position of the Heart Within the Chest, Am. J. M. Sc. **192**: 556, 1936.
4. Katz, L. N., and Korey, H.: Manner in Which Electric Currents Generated by the Heart Are Conducted Away, Am. J. Physiol. **111**: 83, 1935.
5. Lindner, E., and Katz, L. N.: The Relative Conductivity of the Tissues in Contact With the Heart. Observations on Animals With Closed Chests, Am. J. Physiol. **125**: 625, 1939.

CLINICAL CHEMISTRY

PLASMA ALBUMIN, GLOBULIN, AND FIBRINOGEN IN HEALTHY INDIVIDUALS FROM BIRTH TO ADULTHOOD*

II. "NORMAL" VALUES

VIRGINIA TREVORROW, PH.D., MARGARET KASER, PH.D., JEAN PATON PATTERSON, M.S., AND ROBERT M. HILL, PH.D.
DENVER, COLO.

THE literature records several studies of the plasma proteins in "normal" individuals. These values are not wholly adequate, since most of the studies have been made upon small groups of persons, chiefly adults, whose state of health in many cases is open to question. There is a great paucity of data with respect to plasma protein values in infancy and early childhood. In addition to this lack of adequate material, the existing studies have been based upon different methods of analysis which do not give comparable results. The many and varied functions attributed to the different protein fractions of the blood plasma have made it increasingly important that a quantitative study be undertaken to determine the amount of albumin, globulin, and fibrinogen present in the blood of healthy individuals. Such a study also furnishes the necessary foundation for the interpretation of findings in pathologic conditions. We have determined the concentration of the protein fractions in the plasma of 547 healthy persons by means of a salting-out separation of the different proteins, followed by Kjeldahl analysis. The details of our analytical procedure have been given in a previous publication.[1]

MATERIAL USED

The subjects for this study were obtained from various sources. A few of the children were personal friends of ours, or children of the Child Research Council. The majority of the babies and younger children were from maternity hospitals or Homes at which they received regular medical supervision and in which the dietary was considered to be adequate. The adolescents were students at West Denver High School and were examined and questioned by a physician and one of us when the blood was taken. The adults were medical students and laboratory workers. In every case an attempt was made to rule out those who might present a clinical or subclinical variation in any of the protein fractions as a result of recent immunization, malnutrition, dehydration,

*From the Child Research Council and the Department of Biochemistry, University of Colorado School of Medicine, Denver.
Received for publication, October 20, 1941.

chronic or acute disease, or recent injury. Samples were not taken from persons presenting a history of mild illness within the preceding two weeks, or of immunization, severe infection, or contagion within the preceding three months. With two exceptions, the subjects under one year of age were all considered to have been full-term babies. Two babies, one 3 months old and the other 6 months old when the blood was taken, were probably premature at birth. A few persons who had minor injuries, such as blisters or scratches of the hands or feet, were included. A Wassermann test was done on the serum of each child from the institutions, and those few with a positive test have been omitted from the present study. The majority of the children had also been tested with tuberculin at regular intervals while in the Homes. A few (eight) positive tests were obtained, but inasmuch as these showed no clinical or x-ray evidence for active tuberculosis, they have been included.

It is conceivable that each disease to which a child becomes immunized might leave a permanent record of some increase in the total plasma globulin, since the antibodies are found in this fraction. Complete histories were not available on the persons of this study, and therefore, no attempt has been made to classify them on this basis.

Most of the blood samples were taken during the morning. With older children and adults, the majority of the samples were obtained from one-half to two and one-half hours after the usual breakfast. Twenty-six analyses on children between 3 and 9 years of age were made on bloods taken after a fast of twelve hours. No consistent difference was found in any of the protein fractions between the children who had fasted and other children of the same age. Similar findings have been reported by other workers.[2-7] With the small infants, blood samples were also obtained at varying periods of time after feeding. No separation has been made between those who were entirely breast fed and those who received supplemental feedings. In the period from the third day of life to the end of the first month, 90 babies were known to be entirely breast fed, and 15 received one or more supplemental bottle feedings each day. After the first month most of the babies were wholly or partly bottle fed. The small number of bottle-fed babies in the first month of life and of breast-fed babies in the older age groups makes it impossible for us to say with certainty whether the type of feeding is a factor in determining the plasma protein level in infancy; however, no definite association between the type of feeding and the levels of the different protein fractions has been observed in our series. This is in contrast to the work reported by Ujsaghy,[8] in which lower values for the total plasma protein were found in breast-fed infants as compared with artificially-fed infants of the same age.

Blood samples were taken from the external jugular, the femoral, or the scalp veins in the small infants, and from the arm or hand veins in the larger children and adults. In taking the blood, a minimum amount of stasis was used. The blood sample (2 to 4 c.c.) was placed immediately in a tube containing a slight excess of dry heparin (about 0.5 mg.), mixed, and centrifuged in a stoppered tube as quickly as possible. The plasma was separated usually within one hour from the time the blood was taken, and in all cases, within three hours.

RESULTS

Variation in the plasma concentration of the different protein fractions has been determined within groups of similar subjects. In addition, we have attempted to determine the association, if any, between the different protein concentrations and the factors of age, sex, and season of the year. The series of 566 determinations on 547 persons is made up of 303 determinations on males and 263 on females. These are distributed equally throughout the entire age sequence, except in the range above 20 years, in which the females are predominant; and at 11 years and below 6 months, where the number of males is in excess. The blood samples have been separated into two groups according to the season in which they were done: "summer" extending from April 1 through October 31, and "winter," from November 1 through March 31. In the age period from one month to 6 months, the grouping according to season is uneven. From 6 months to 12 years the analyses are more evenly divided between winter and summer for each age interval and for each sex. For the group from 12 to 20 years, most of the analyses were done during the winter, and from 20 to 40 years, in the summer. The complete distribution of the analyses is summarized in Table I.

TABLE I

DISTRIBUTION OF ANALYSES ACCORDING TO SEASON OF YEAR, SEX, AND AGE OF PERSONS

AGE	NUMBER OF ANALYSES			
	MALES		FEMALES	
	WINTER	SUMMER	WINTER	SUMMER
Birth to 15 days	27	18	16	13
15 days to 31 days	19	15	8	11
1 month to 6 months	34	9	20	6
6 months to 4 years	20	26	22	18
4 years to 8 years	16	25	14	29
8 years to 12 years	22	26	12	25
12 years to 20 years	25	15	34	8
20 years to 40 years	2	4	6	21

Limitations of space prevent a detailed presentation of data; however, these have been analyzed by accepted statistical procedures (Mainland[9]) and the significant findings are given.

Albumin.—The albumin fraction shows no significant variation between the sexes, nor with the height, weight, or body surface of the subjects, but it does vary with age and with the season of the year. During the first month of life the albumin is low, the mean value being 3.79 Gm. per 100 c.c., with a standard deviation of ±0.33 Gm. There is no apparent change with age at this early period, but the variation between different babies of the same age is large. At about one month a rise in the albumin with increasing age becomes apparent. This continues until the "adult" level is reached, sometime between 6 months and 1 year, after which there is no further significant change with age. As pointed out, the babies in our series were predominantly breast fed in the first month of life and bottle fed after this age. It, therefore, appears possible that the increasing plasma albumin concentration may be associated with the change in diet and may not be entirely referable to age. A consideration of the 90

breast-fed and 15 bottle-fed infants under one month of age gives no indication of a relationship between the plasma albumin level and the type of feeding; however, a larger group of infants must be studied before such a relationship can be determined definitely. In our series there is an apparent rise in the plasma albumin level between the ages of 13 and 20 years old. This may result from the fact that this group of analyses was done during the winter, and the rise in values may be ascribed to seasonal variation rather than to a change with age. The mean "adult" level is 4.70 Gm. per 100 c.c., with a standard deviation of ±0.32 Gm.

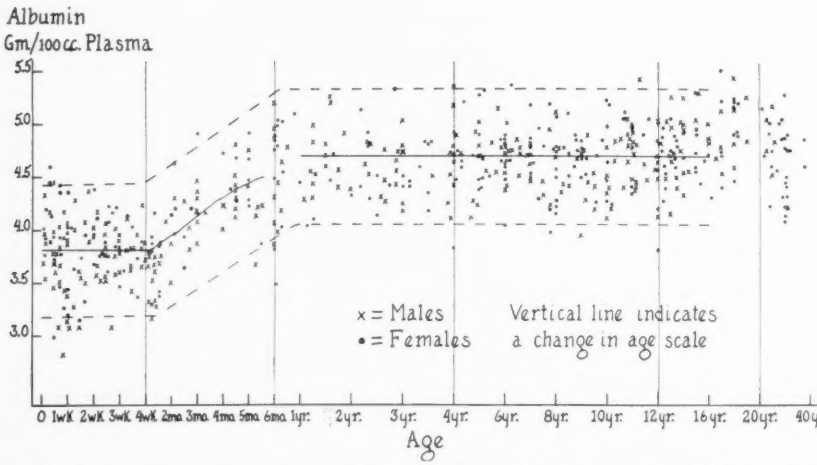


Chart 1.—A scatter diagram of albumin, expressed as grams per 100 c.c. of plasma, referred to age. The unbroken line indicates mean values; the region between the broken lines is that included within plus and minus two standard deviations from the mean.

We have determined the trend of values during the period of change. This is shown graphically in Chart 1, together with the values for both younger and older persons. It can be seen that the spread covered by two standard deviations, including about 95 per cent of the cases, is very large for all ages. This spread is made up of two components, the true biologic variation and the variation caused by chance errors in the analytic procedure. The true biologic variation has been calculated as follows:

$$\begin{aligned} \text{S.D.}_{\text{B}} &= \sqrt{(\text{S.D.}_{\text{T}})^2 - (\text{S.D.}_{\text{M}})^2} \\ &= \sqrt{0.32395^2 - 0.07705^2} \\ &= \sqrt{0.099007} \\ &= \pm 0.314 \end{aligned}$$

The symbol "S.D." represents the standard deviation, and the subscript letters "B," "T," and "M" refer to the deviation characterized as biologic, total, and measurement, respectively. It is seen that the true variation between individuals is large, even after allowing for the increment caused by errors of measurement. By means of longitudinal studies we hope to determine whether this wide spread indicates that the plasma albumin concentration varies widely in a given person from time to time, or maintains itself within the individual, at a high or low level in relation to other persons.

The albumin analyses have been studied with respect to seasonal variation. In the series of 127 babies up to one month of age, there is no significant difference in the plasma albumin level at different seasons of the year. For persons over 3 years of age, 142 winter samples give a mean value of 4.83 ($S.D._T = \pm 0.30$), and 167 summer samples show a mean of 4.59 ($S.D._T = \pm 0.31$). Although this difference is not great, the probability that it would occur by chance alone is much less than 1 in 100. Chart 2 shows the frequency distribution of albumin values in the two groups. With the data available we have not been able to determine at what point between one month and 3 years of age the seasonal variation appears.

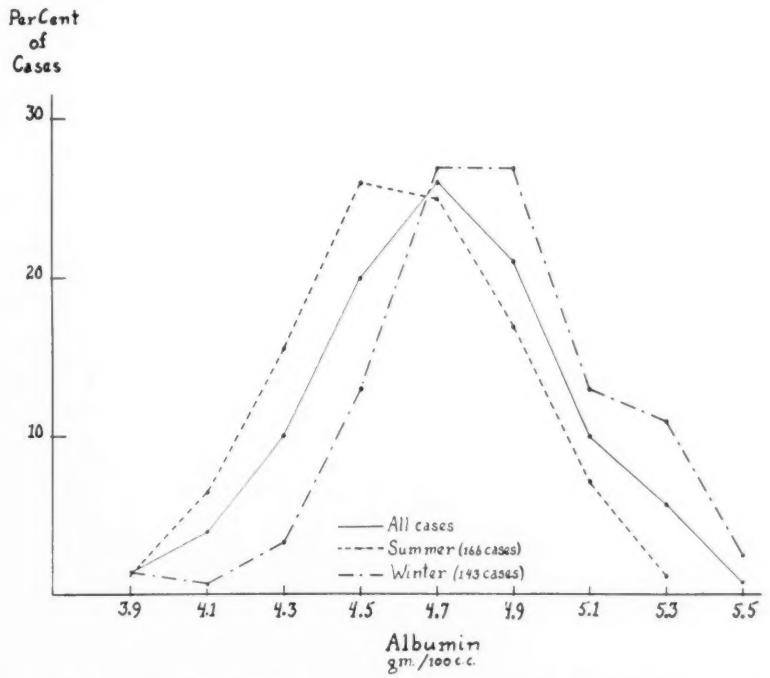


Chart 2.—Winter and summer frequency polygons of albumin values in 309 analyses on individuals over 3 years of age.

Bazett, Sunderman, Doupe, and Scott[10] have presented evidence to show that during the transition from winter to summer there is an increase in blood volume in human subjects and a corresponding decrease in total plasma protein concentration. As the warmer temperature is maintained, the proteins increase until the initial level is reached. During the transition from summer to winter, the reverse set of changes takes place. It might at first seem that this type of mechanism could account for the seasonal variation in albumin which we have observed, since our "summer" group would, to a large extent, include the transition from winter to summer, and the "winter" group would demonstrate the opposite change. However, since the globulin and fibrinogen have not shown a seasonal variation similar to that observed in the albumin fraction, the picture is more complex than one of simple dilution and concentration of the plasma.

Yosida[11] has studied rabbits with respect to the seasonal variation in certain plasma constituents. Although it is difficult to interpret his findings in terms of absolute values for the protein constituents, the figures given should be comparable within themselves. His results on this species of animal show a higher albumin during the summer months than during the winter. This is in direct contradiction to our results in which the lower albumin values occurred during the summer. It seems possible that different species of animals might respond differently to temperature changes. It is also possible that the variation which we have observed may be the result of changes in diet and living habits rather than temperature changes, whereas in the case of rabbits, the diet, activity, and living conditions are more subject to control.

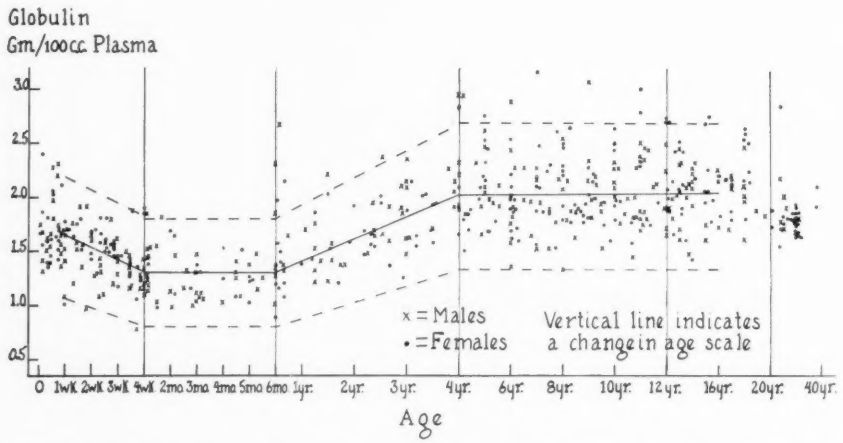


Chart 3.—A scatter diagram of globulin, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

Globulin.—The plasma globulin concentration has been found to vary with age, as shown in Chart 3, but it does not vary with sex or size of the person, nor with the season of the year. At birth, and for the first week of life, the globulin is present in a mean concentration of 1.66 Gm. per 100 c.c., with a standard deviation of ±0.29. Between the first and the fifth week the value falls to 1.31 Gm. per 100 c.c. ($S.D._T = \pm 0.25$), where it is maintained until the age of about 6 months. At about 6 months a gradual rise in globulin concentration becomes apparent, and continues to an age of about 4 years, at which time the "adult" level of 2.03 Gm. per 100 c.c. ($S.D._T = \pm 0.34$) is reached. After 4 years, up to 20 to 40 years, there is no significant change in the globulin level. In Chart 3 there appears to be a fall in the globulin in the period between 20 and 40 years. This is probably not a real change, but it may result from the fact that 15 determinations in this age group were obtained on two individuals who have maintained a consistently low globulin over a period of many months.

As was the case with the albumin, the greater part of the variation in values at any age level is apparently a true biologic variation, and not the result of chance errors of analysis. For all persons over 5 years of age the mean globulin concentration is 2.03 Gm. per 100 c.c.; the standard deviation representing biologic variation ($S.D._B$) is ±0.34.

The distribution of values shows a definite skew toward the higher globulin concentrations, as shown in Chart 4. Similarly, there are more above the area covered by plus or minus two standard deviations than there are below it, as can be seen in Chart 3. There is no known explanation for this fact; however, it seems possible that in spite of the care exercised in the selection of subjects, a few may have been included who show an elevated globulin as a result of infection or immunization. It is known[12-15] that the development of antibodies is frequently accompanied by an increase in some fraction of the total globulin, but little is known of the duration of this hyperglobulinemia.

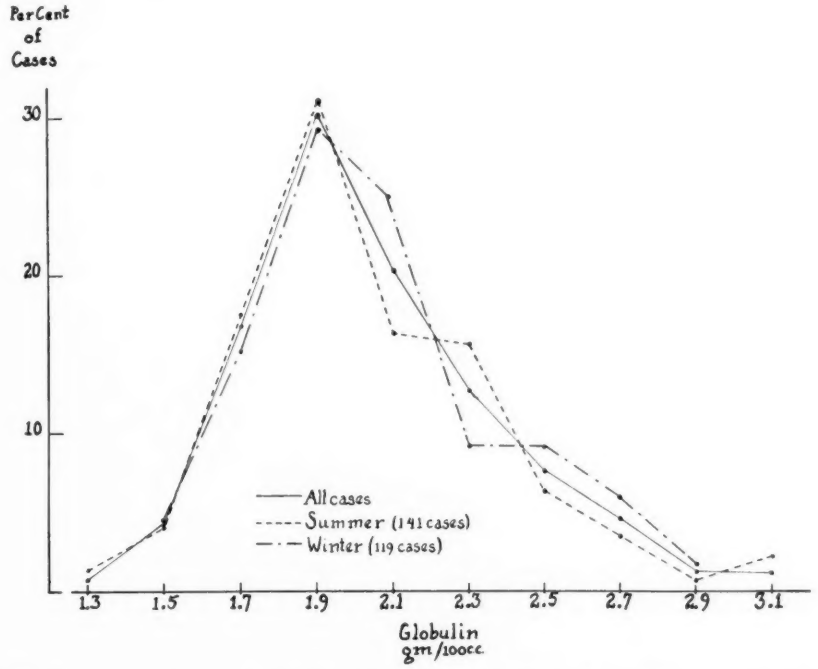


Chart 4.—Frequency polygon of globulin values in 260 analyses on persons over 5 years of age.

Fibrinogen.—The most striking feature of the findings with respect to fibrinogen is a wide scatter of the values in all groups. Chart 5 shows the distribution of values for both sexes graphed with respect to age. From the chart it will be observed that there is a slight tendency toward a higher mean value and greater variation at birth than in the older age groups. This may or may not be a real change; in any event it is probably too small to be of clinical significance, except in so far as it may indicate instability of the plasma fibrinogen concentration in the first weeks of life. After one month there is no significant change with age in the mean level of plasma fibrinogen. This value is 0.21 Gm. per 100 c.c., with a standard deviation (total) of ± 0.0590. As was the case with the albumin and globulin fractions, the spread in values seems to be due to a true biologic variation, the standard deviation corrected for chance errors of analysis being 0.0585.

In the period up to 5 years of age there is no significant difference between the sexes in the fibrinogen level. After this age the males tend toward lower values, the mean for 123 males being 0.201 ($S.D._T = \pm 0.054$), as contrasted with a mean of 0.218 ($S.D._T = \pm 0.058$) for 136 females. Although this difference is not great, the probability that it would occur by chance alone is between 1 and 2 in 100.

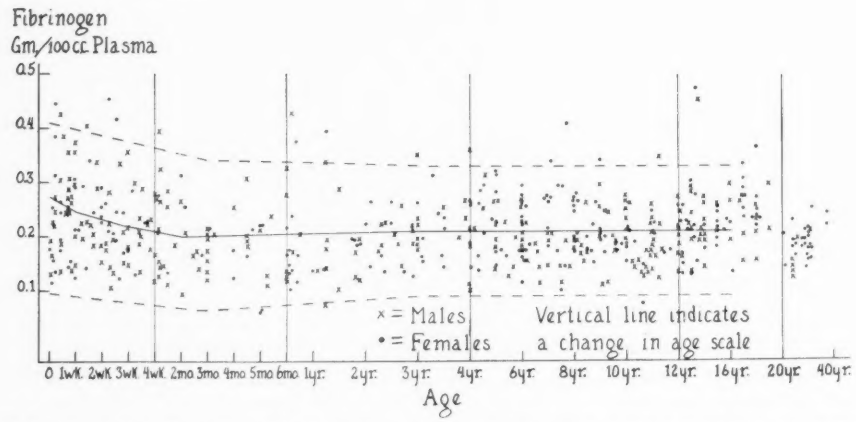


Chart 5.—A scatter diagram of fibrinogen, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

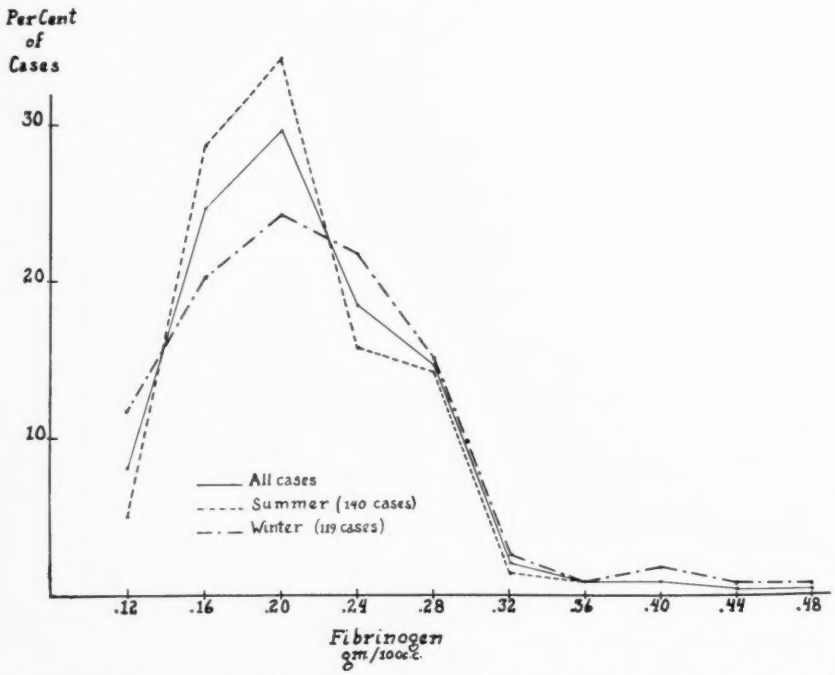


Chart 6.—Frequency polygon of fibrinogen values in 259 analyses on persons over 5 years of age.

The distribution of fibrinogen values about the mean, as shown in Chart 6, shows a skew toward the higher values somewhat similar to that observed with the globulin. This similarity raises a question of correlation between the two

fractions. Chart 7 shows the fibrinogen values in relation to each corresponding globulin value for all analyses on persons over 5 years of age. Although a slight correlation is present, it is to be noted that, with one exception, the fibrinogen values above two standard deviations do not occur in the same persons as those showing globulin values above the two standard deviation range. It seems likely, therefore, that the factor, or factors, producing the skew in the globulin distribution are not the same as those involved in the fibrinogen distribution.

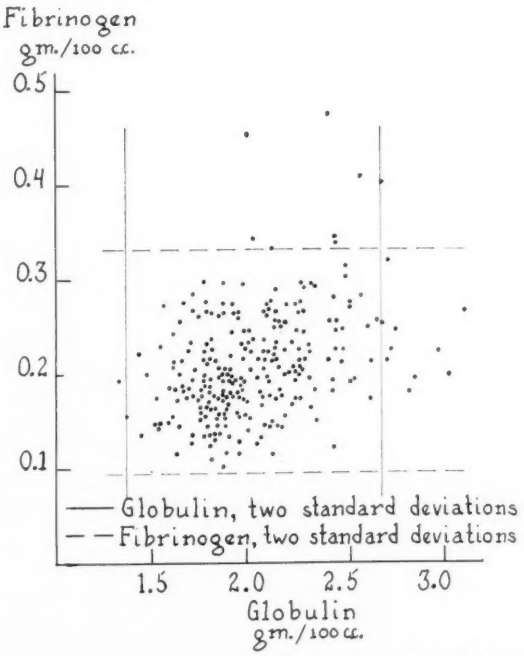


Chart 7.—A scatter diagram of fibrinogen concentration referred to the corresponding globulin concentration in 259 samples of plasma from individuals over 5 years of age.

Ham and Curtis[16] have reviewed the literature with respect to fibrinogen values that have been reported, and have studied this factor in a series of adults under varying physiologic and pathologic conditions. In their discussion these authors state, "Normal pregnancy is the only 'physiological' condition associated with increased fibrinogen concentrations to values of from 350 mg. to 450 mg. *Diagnostically*, therefore, an abnormally elevated plasma fibrinogen concentration in the absence of pregnancy is laboratory evidence for an organic disease process which may be present at the time of observation or which may have terminated several days or weeks before." Our series shows plasma fibrinogen concentrations above 0.4 Gm. per 100 c.c. in 4 of 259 persons over 5 years of age. At the time of the analyses these persons, as stated previously, had had no minor illness (mild colds) in the preceding two weeks, and no serious illness with fever during the preceding three months. One of the four had recovered from pneumonia three and one-half months before the determination was done and may have had an elevated fibrinogen which would extend over this period of time. The other three children had not shown symptoms of any acute or

chronic disease, nor had they been immunized against any disease during an eight-month period preceding the plasma protein determination. One must conclude, therefore, that (1) the findings of Ham and Curtis do not apply to children; (2) allowance must be made for an occasional "normal" plasma fibrinogen concentration above the limits set by these authors; or that (3) the fibrinogen elevation following disease is more prolonged than these authors would lead one to believe. With the data at hand we have been unable to determine which of these three possibilities is the correct interpretation of our results.

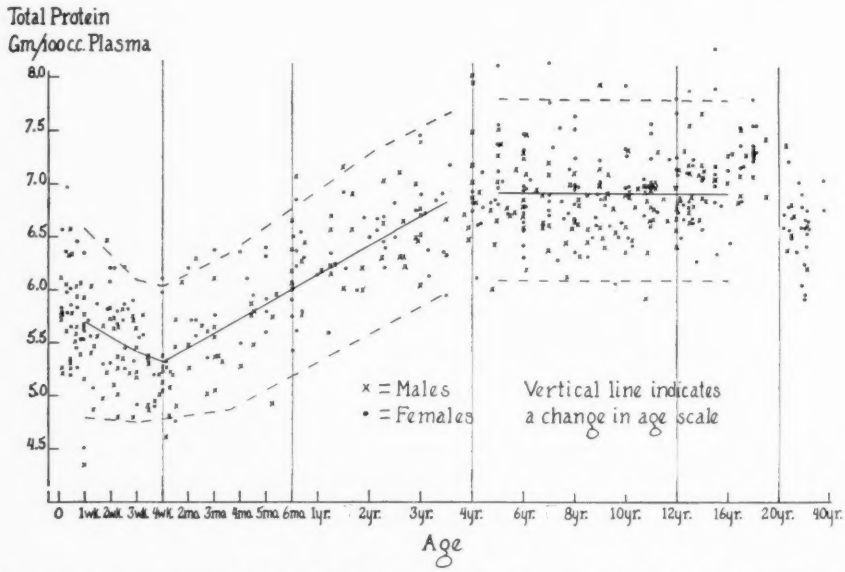


Chart 8.—A scatter diagram of total protein, expressed as grams per 100 c.c. of plasma, referred to age (see legend of Chart 1).

Total Protein.—The total protein is frequently determined without separate analysis of the different fractions. This practice leaves much to be desired, since most of the functions of the proteins may be attributed primarily to one or another of the fractions rather than to their combined, total concentration.[17] On the other hand, the determination of total protein is a relatively simple procedure and gives some information of value to the clinician. The total protein reflects the difference between the sexes with respect to fibrinogen and the seasonal variation observed in the albumin; however, the most significant variation is that occurring with age. At birth the total protein concentration is 5.70 Gm. per 100 c.c., with a standard deviation (total) of ±0.45 Gm. per 100 c.c. During the first month of life the value falls to 5.33 Gm. per 100 c.c. ($S.D._T = \pm 0.37$), after which there is a gradual rise until the adult level of 6.94 ($S.D._T = \pm 0.47$) is reached at about 4 years of age. These changes are shown graphically in Chart 8.

DISCUSSION

Of the many methods used in the study of plasma protein concentrations, the one based upon Kjeldahl analysis has best survived the test of time. This is because the results obtained with this method may be more accurately repro-

duced, and are less subject to errors caused by extraneous factors which may be present, especially in pathologic conditions (see Chorine,[7] and Myers and Muntwyler[20]). In the analysis of fibrinogen the values obtained by different methods may be more comparable. Our discussion of albumin and globulin concentrations will be limited to the studies in which a salting-out procedure has been used to separate the fractions, and the analysis has been based upon Kjeldahl nitrogen determination. In some instances, workers have used a factor other than 6.25 to convert the nitrogen content to protein. These figures have been recalculated using 6.25 as the factor in order that all the results may be expressed in the same terms. We do not wish to imply a belief that the different proteins contain exactly 16 per cent nitrogen; however, this nitrogen content is most uniformly assumed, and the protein concentrations calculated upon this basis may be easily corrected at some later date if it is shown that their nitrogen content varies significantly from this value.

Table II gives a summary of albumin and globulin values reported in the literature. Some of these workers have used serum; others have used plasma. In the latter group both heparin and oxalate have been employed as anticoagulants, and in two instances no statement is made concerning the anticoagulant. Of the six workers who have analyzed plasma, only two give values for fibrinogen. In three papers the sum of the mean values for albumin and globulin approximates the mean total protein, and it is, therefore, assumed that the so-called "globulin" includes both globulin and fibrinogen. In the report of Muntwyler, Way, Binns, and Myers,[27] the mean total protein is greater than the sum of the albumin and globulin, and we have, therefore, assumed that the fibrinogen was determined separately. A variety of salts has been used to separate the albumin and globulin. We have not attempted to analyze the reported values on this basis, but we have included all papers in which a salting-out procedure was employed, irrespective of the salt used. In none of the work to be discussed has any mention been made of the season of the year in which the analyses were done. Seasonal variation in the albumin fraction might, to some extent, account for the disagreement among different workers with respect to this fraction.

The data summarized in Table II cover analyses on 102 adults and 199 children from birth to 15 years. For the entire group of adults the mean albumin concentration is about 4.6 Gm. per 100 c.c., with a range from the low of 3.3 reported by Lewinski[21] to a high of 5.65 reported by Bruckman, D'Esopo, and Peters.[26] This mean value is in relatively good agreement with the mean albumin concentration of 4.7 Gm. per 100 c.c., which we have found in older children and adults, and only the low values reported by Lewinski[21] and by Linder, Lundsgaard, and Van Slyke[22] fall outside the three standard deviation range covered by our series. On the other hand, the mean values reported by the different workers for their respective small groups of persons vary widely with respect to each other and in comparison with our mean.

The globulin analyses reported in the literature cover 54 adults, with a combined mean of about 2.0 Gm. per 100 c.c. and a range from 1.32 to 3.79. This mean value is the same as we have found for older children and adults, as is the total range, except for one high value (3.79) reported by Lewinski.[21]

TABLE II

PLASMA PROTEIN VALUES REPORTED IN THE LITERATURE IN WHICH THE METHOD OF ANALYSIS IS SIMILAR TO THAT EMPLOYED IN THIS STUDY

AUTHOR DATE REFERENCE	NUMBER OF CASES	AGE	ALBUMIN‡	GLOBULIN‡	FIBRINOGEN‡	REMARKS
Lewinski (1903)[21]	3	Adult	3.30-4.47	2.36-3.79	0.28-0.50	Oxalated plasma
Linder, Lundsgaard, and Van Slyke (1924)[22]	7	Adult	3.62-4.9 (4.11)	2.45-2.89 (2.61)	In globulin?	Oxalated plasma
Salvesen (1926)[23]	16 males	Adult	3.95-5.24 (4.44)	1.96-3.16 (2.58)	In globulin?	Oxalated plasma
	16 females	Adult	3.77-4.80 (4.55)	2.18-3.55 (2.68)	In globulin?	Oxalated plasma
Wiener and Wiener (1930)[24]	20	Adult	4.20-5.00 (4.60)	1.50-1.90 (1.70)	Male 0.22-0.28 (0.25)	Anticoagulant not given
					Female 0.25-0.33 (0.28)	Plasma used
Moore and Van Slyke (1930)[25]	9	Adult	4.0-4.5 (4.3)	(2.8)	In globulin?	Heparinized plasma
Bruckman, D'Esopo, and Peters (1930)[26]	13 males	Adult	4.37-5.65 (5.06)	1.32-2.91 (1.89)	------	Serum
	8 females	Adult	4.71-5.17 (4.98)	2.02-3.22 (2.62)	------	Serum
Muntwyler, Way, Binns, and Myers (1933)[27]	10 males	Adult	(4.84)	(1.88)	?	Anticoagulant not given Plasma used
Darrow and Cary (1933)[28]	20	Newborn over 3 days	(3.73) S.D. -0.38	(1.78) S.D. -0.45	------	Serum
	16	5-6 mo.	(4.28) S.D. -0.38	(2.01) S.D. -0.34	------	Serum
Rennie (1934)*[29]	6	3-6 mo.	(4.67)	(1.84)	------	Serum
	4	½-1 yr.	(4.76)	(1.99)	------	
	5	1-1½ yr.	(5.26)	(2.27)	------	
	20	Over 1½ yr.	(5.02)	(2.39)	------	
Rennie (1935)†[30]	22	3-23 mo.	4.1-5.9	1.13-2.82	------	Serum
	24	2½-11 yr.	4.0-5.5	1.4-3.0	------	
Dodd and Minot (1936)[31]	16	Birth to 3 mo.	2.6-4.2 (3.66)	(1.78)	------	Serum
	34	3 mo.-2 yr.	3.6-4.8 (4.28)	(1.91)	------	
	32	2-15 yr.	3.9-5.2 (4.65)	(2.38)	------	

*Patients have "no apparent abnormality except mild rickets."
†Patients are afebrile convalescents.
‡Numbers in parentheses indicate mean values.

In contrast to this, the mean value of 2.6 Gm. per 100 c.c. for globulin plus fibrinogen, reported in the literature for 48 adults, is distinctly above the sum of our mean values for the two fractions.

Serum analyses have been reported in the literature for 199 children. These results have not been presented for each individual, but for age groups which frequently include periods where we have found the concentration of one of the

fractions to be changing. Thus the mean values for these groups cannot be exactly compared with our series, since we do not know the distribution of persons within the groups that have been reported. In general, the albumin concentrations which have been reported fall well within the range of values covered by our series for corresponding age periods. A notable exception to this is the high mean albumin level of 5.07 Gm. per 100 c.c. reported by Rennie[29] in 1934 for 25 children over 1 year old. This is in contrast to our mean of 4.7 for all children over 1 year old, or the mean of 4.83 for the analyses done during the winter months on children of this age group.

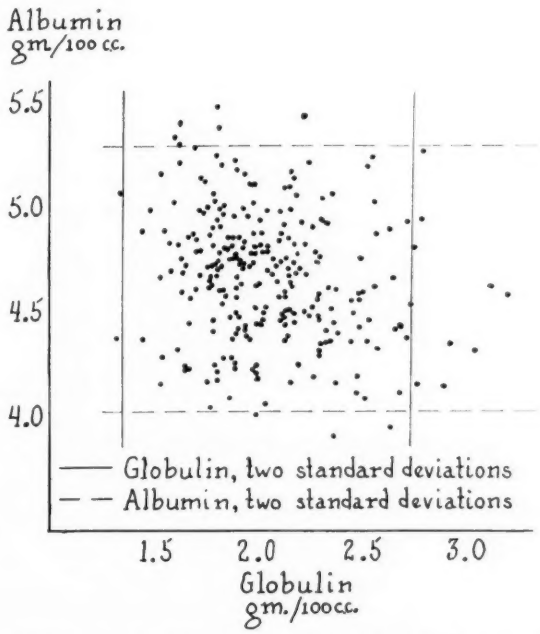


Chart 9.—A scatter diagram of albumin concentration referred to the corresponding globulin concentration in 262 samples of plasma from individuals over 5 years of age.

The serum globulin values which have been reported for children tend to be higher than those of our series at corresponding age levels. A possible explanation of this discrepancy might be that some of the children used by other workers, although not acutely ill when the blood was taken, may have shown a mild hyperglobulinemia as a result of recent infection or immunization.

Ham and Curtis[16] have reviewed the literature with respect to plasma fibrinogen concentration in normal persons. In 193 determinations on adults reported by these authors, and in the work reviewed by them, the fibrinogen values fall between 0.190 and 0.380 Gm. per 100 c.c., with a mean of about 0.250. These results are essentially the same as those given in Table II, as reported by Lewinski,[21] and by Wiener and Wiener.[24] Our mean value for persons between 3 and 20 years is slightly below this, and our range is somewhat wider, including many values below those found in the literature, and a few which are higher. We have been unable to find any extensive literature covering the plasma fibrinogen concentration in early childhood or infancy. It is

quite possible that our results on persons from 3 to 20 years of age would not represent the values to be expected in persons from 16 to 61 years, the age range studied by other workers. In our series there is an apparent rise in the fibrinogen in the period from 12 to 20 years. This is offset by the low values observed between 20 and 40 years, and the mean for the entire group beyond 12 years is the same as that for children from 3 to 12 years of age. A real tendency toward increasing fibrinogen concentration after puberty can be established only by studies on a larger group of persons. The results reported in the literature may indicate that such an increase does occur.

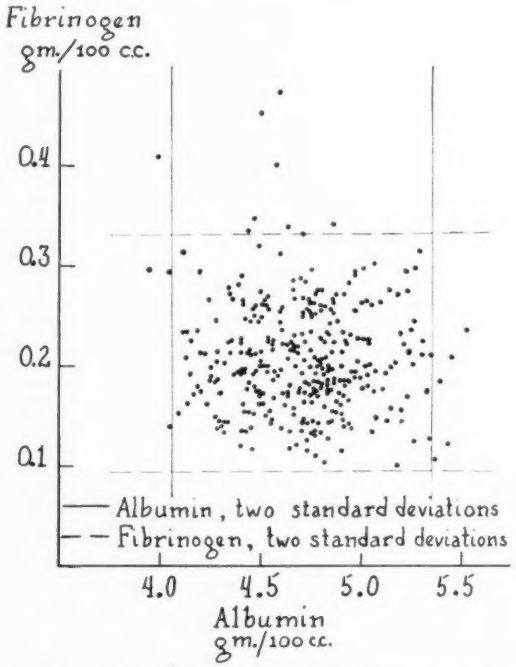


Chart 10.—A scatter diagram of fibrinogen concentration referred to the corresponding albumin concentration in 259 samples of plasma from individuals over 5 years of age.

We are not satisfied that the ratios of albumin to globulin have sufficient significance to justify including these values in our tables. It is true that plasma and serum protein determinations are frequently reported in terms of the total protein concentration and the ratio of albumin to globulin, the A./G. ratio. With serum analysis this method of recording, although unnecessarily obscure, gives a complete picture, for one can calculate the more significant individual values for albumin and globulin if the total protein and the A./G. ratio are given. When plasma is used, however, confusion arises because some workers include the fibrinogen with the globulin in computing the A./G. ratio, while others do not.

Interpretation of the results of plasma protein determinations, from either the physiological or clinical point of view, is based largely upon the concentration of each separate fraction, with its independent functions, and not upon the ratios between the different parts. Furthermore, in our work with healthy persons, the three major protein fractions have been found to vary independently

of each other, as shown in Charts 7, 9, and 10. Therefore, we believe that the albumin, globulin, and fibrinogen should be treated as separate entities (or systems), and that it is more satisfactory to record in the literature the actual values of the separate protein fractions rather than a ratio between any of the constituent parts.

Boissevain[18, 19] has recently reported that serum albumin greatly promotes, and serum globulin inhibits, the growth of the tubercle bacillus on artificial media. Should the two proteins be shown to have an antagonistic action in tuberculosis, the A./G. ratio would become of importance in so far as these patients are concerned.

SUMMARY AND CONCLUSIONS

1. A total of 566 analyses of plasma albumin, globulin, and fibrinogen have been made on 547 healthy persons from birth to 39 years of age.

2. The protein fractions have been separated by salting-out with sodium sulfate and analyzed by a micro-Kjeldahl procedure.

3. Each of the protein fractions undergoes a change with age in early infancy and childhood. These changes have been defined.

4. The plasma albumin concentration in persons over 3 years of age is higher in the winter than in the summer months. This seasonal variation does not appear in the other fractions.

5. In persons over 5 years of age the plasma fibrinogen tends to be higher in females than in males.

6. In normal persons the albumin, globulin, and fibrinogen vary independently.

7. No correlation has been found between any of the protein concentrations and the time elapsing after the ingestion of food.

8. There is no correlation between any of the protein concentrations and the height, weight, or body surface of the individuals.

REFERENCES

1. Hill, R. M., and Trevorrow, V.: Plasma Albumin, Globulin, and Fibrinogen in Healthy Individuals From Birth to Adult Life, I. A System of Microanalysis, J. LAB. & CLIN. MED. **26**: 1838, 1941.
2. Reiss, E.: Die refraktometrische Blutuntersuchung und ihre Ergebnisse für die Physiologie und Pathologie des Menschen, Ergebn. d. inn. Med. u. Kinderh. **10**: 531, 1913.
3. Alder, A.: Die physiologischen Schwankungen des Mischungsverhaltnisses von Albumin und Globulin im menschlichen Blutserum, Deutsches Arch. f. klin. Med. **126**: 61, 1918.
4. Rowe, A. H.: The Effect of Muscular Work, Diet, and Hemolysis on the Serum Proteins. Together With Comment on the Technic and Clinical Usefulness of Robertson's Microrefractometric Method, Arch. Int. Med. **19**: 499, 1917.
5. Kerr, W. J., Hurwitz, S. H., and Whipple, G. H.: Regeneration of Blood Serum Proteins. I. Influence of Fasting Upon Curve of Protein Regeneration Following Plasma Depletion, Am. J. Physiol. **47**: 356, 1918-19.
6. Howe, P. E.: The Function of the Plasma Proteins, Physiol. Rev. **5**: 439, 1925.
7. Chorine, V.: Influence des anticoagulants sur le dosage des elements du sang, Ann. Inst. Pasteur **63**: 213, 1939.
8. Ujsághy, P.: Untersuchungen über Eiweissvorrate im Organismus des Sauglings und des Kindes, Ztschr. f. Kinderh. **62**: 483, 1941.
9. Mainland, D.: The Treatment of Clinical and Laboratory Data, London, 1938, Oliver & Boyd.

10. Bazett, H. C., Sunderman, F. W., Doupe, J., and Scott, J. C.: Climatic Effects on the Volume and Composition of Blood in Man, Am. J. Physiol. **129**: 69, 1940.
11. Yosida, N.: Studium über verschiedene Einflusse auf Veranderung des Eiweisskorpers und Phosphatids des Blutplasmas, besonders auf ihre gegenseitige Beziehung, J. Biochem. **31**: 243, 1940.
12. Bjorneboe, M.: The Specific Protein in Rabbit Antipneumococcal Serum and Its Relation to the Increase of Serum Protein During Immunization, J. Immunol. **37**: 201, 1939.
13. Bing, J.: Further Investigations on Hyperglobulinemia, Acta med. Scandinav. **103**: 547, 565, 1940.
14. Marrack, J. R.: The Chemistry of Antigen and Antibodies, Medical Research Council, Special Report No. 230, 1938.
15. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A.: Electrophoretic Patterns of Normal and Pathological Human Blood Serum and Plasma, J. Exper. Med. **70**: 399, 1939.
16. Ham, T. H., and Curtis, F. C.: Plasma Fibrinogen Response in Man; Influence of Nutritional State, Induced Hyperpyrexia, Infectious Disease and Liver Damage, Medicine **17**: 413, 1938.
17. Bennhold, H., Kylin, E., and Rusznyak, S.: Die Eiweisskörper des Blutplasmas, Dresden and Leipzig, 1938, Theodor Steinkopff.
18. Boissevain, C. H.: Growth Promotion of the Tubercle Bacillus by Serum Albumin, Proc. Soc. Exper. Biol. & Med. **44**: 110, 1940.
19. Boissevain, C. H.: Personal communication.
20. Myers, V. C., and Muntwyler, E.: Chemical Changes in the Blood and Their Clinical Significance, Physiol. Rev. **20**: 1, 1940.
21. Lewinski, J.: Beobachtungen über den Gehalt des Blutplasmas an Serumalbumin, Serumglobulin und Fibrinogen, Arch. f. d. ges. Physiol. **100**: 611, 1903.
22. Linder, S. C., Lundsgaard, C., and Van Slyke, D. D.: The Concentration of the Plasma Proteins in Nephritis, J. Exper. Med. **39**: 887, 1924.
23. Salvesen, H. A.: Plasma Proteins in Normal Individuals, Acta med. Scandinav. **65**: 147, 1926-27.
24. Wiener, H. J., and Wiener, R. E.: Plasma Proteins, Arch. Int. Med. **46**: 236, 1930.
25. Moore, N. S., and Van Slyke, D. D.: The Relationships Between Plasma Specific Gravity, Plasma Protein Content and Edema in Nephritis, J. Clin. Investigation **8**: 337, 1930.
26. Bruckman, F. S., D'Esopo, L. M., and Peters, J. P.: The Plasma Proteins in Relation to Blood Hydration. IV. Malnutrition and the Serum Proteins, J. Clin. Investigation **8**: 577, 1930.
27. Muntwyler, E., Way, C. T., Binns, D., and Myers, V. C.: Plasma Protein and Plasma Colloid Osmotic Pressure in Pathological Conditions With Special Reference to Occurrence of Edema, J. Clin. Investigation **12**: 495, 1933.
28. Darrow, D. C., and Cary, M. K.: The Serum Albumin of Newborn, Premature and Normal Infants, J. Pediat. **3**: 573, 1933.
29. Rennie, J. B.: Nephritis in Infancy, Arch. Dis. Child. **9**: 295, 1934.
30. Rennie, J. B.: A Note on the Serum Proteins in Normal Infants and Children, Arch. Dis. Child. **10**: 415, 1935.
31. Dodd, K., and Minot, A. S.: The Occurrence of Moderately Reduced Serum Albumin in Five Hundred Children in a Southern Clinic, J. Pediat. **8**: 452, 1936.

BLOOD FROM THE EAR LOBE*

Preliminary Report

G. Brückmann, Ph.D., Tel Aviv, Palestine

IT SEEMS generally accepted that, under standard conditions, practically identical hemoglobin values are obtained from venous and peripheral blood. A closer review of the literature, however, reveals that this view is based in most cases only on comparison of venous blood with blood from the finger tip. Only a few investigators have worked with blood from the ear lobe as representing the peripheral sample, and have found no significant differences for hemoglobin or red blood cells (Bürker,[1] Yarbrough,[2] Foord,[3] Reichel and Monastero,[4] Price-Jones and co-workers[5]).

During hematologic work at this laboratory concerned with the establishment of standard red blood cell values for Palestine and the study of seasonal influences on these values, the usually taken venous samples were—very occasionally—replaced by samples from the ear lobe; in each of these cases, abnormally high hemoglobin figures were observed. A reinvestigation of the question seemed, therefore, advisable, in spite of the fact that it appears to be settled.

EXPERIMENTAL

As mentioned this study was undertaken as a corollary to standard work, which has been carried out along the internationally adopted lines of such investigations and will be described in detail in a forthcoming publication. If not otherwise stated, the subjects comprised healthy adults between 20 and 40 years of age, presenting themselves before breakfast or four hours after a meal. Blood was taken from the cubital arm vein without tourniquet, Liquoid Roche† being used as the anticoagulant, which, like heparin, causes no shrinkage during the hematocrit determinations.

After wiping the finger pulp and the ear lobe with a little ether, the peripheral samples were simultaneously taken by puncture with a spring lancet, care being taken to avoid squeezing or cyanosis. If only hemoglobin was determined, no anticoagulant was used; otherwise approximately 0.5 c.c. of peripheral blood was collected in small vials prepared with the anticoagulant, as described by Andresen.[6]

Hemoglobin was determined spectrophotometrically after reduction with sodium hydrosulfite ($Na_2S_2O_4$) according to Heilmeyer,[7] using a Leitz extinction photometer with screen 570 mμ. This method, which has also been used by Bürker[1] and Williamson,[8] has proved, after standardization, very satisfactory in our hands. To demonstrate the accuracy of the method, the results of 10 *independent* determinations on the same blood sample are given:

*From the Chemical Laboratory, Hadassah Municipal Hospital, Tel Aviv.
†Sodium polyanethole sulfonate.
Received for publication, July 25, 1941.

TABLE I

SIMULTANEOUS HEMOGLOBIN DETERMINATIONS ON VENOUS AND PERIPHERAL BLOOD
(Results in Grams Per Cent)

NO.	NAME	VENOUS	FINGER TIP	EAR LOBE	REMARKS
1	Lan	14.8	14.3	15.9	
2	Bru	15.2	15.2	16.1	
3	Gla	15.8	15.8	17.0	
4	Wal	15.4	15.4	19.3	
5	Kov	16.4	15.7	17.5	
6	Dre	15.0	15.4	16.3	
7	Fis	15.2	15.2	18.3	
8	Loe	11.3	11.3	12.0	Normocytic anemia (pregnant)
9	Mel	15.7	16.1	16.3	
10	Gru	13.4	13.2	16.1	
11	Fei	14.5	13.6	14.5	
12	Ste	13.4	13.4	17.5	
13	Lae	15.2	16.2	14.8	
14	Jac	15.4	14.3	15.4	
15	Bru	13.8	12.5	14.0	
16	Ber	13.8	13.4	15.2	
17	Wei	16.6	16.6	17.7	
18	Fis	14.2	13.6	15.6	
19	Mar	15.7	14.0	17.0	
20	Kar	12.5	11.8	14.0	
21	Jac	14.5		14.7	
22	Kop	10.5		10.8	Macrocytic anemia
23	Dre	16.7		17.2	
24	Lau	11.8		14.0	
25	Bru	12.7		12.9	
26	Mey	9.6		11.4	Microcytic hypochromic anemia
27	Kor	12.5		12.5	
28	Mac	13.4		16.3	
29	Mey	13.4		15.7	
30	Rem	13.1		14.7	
31	Bru	15.1		16.1	
32	Fis	14.2		15.6	
33	Gur	14.4		15.0	
34	Mos	17.6		18.4	
35	Mir	13.2		15.4	
36	Bin	13.6		14.5	
37	Gef	15.0		17.7	
38	Sch	7.5		8.8	Microcytic hypochromic anemia
39	Tra	14.0		15.9	
40	Lev	15.7		17.0	

Mean value: 15.96 grams per cent. Standard deviation: 0.44. Standard error: 0.14. Coefficient of variation: 2.78.

RESULTS

Table I gives the results of the simultaneous determinations on venous and peripheral blood, obtained throughout the year. Ten additional comparisons of venous and finger tip blood are for the sake of brevity not listed in the table, but are included in the statistical evaluation given in Table II.

TABLE II

STATISTICAL EVALUATION OF 30 COMPARISONS OF VENOUS AND FINGER-TIP BLOOD

SOURCE	MEAN VALUE	STANDARD DEVIATION	STANDARD ERROR	COEFFICIENT VARIATION	DIFFERENCE OF MEANS	STANDARD ERROR OF DIFFERENCE
Venous	14.40	2.00	0.365	13.9	−0.25	0.52
Finger tip	14.15	2.06	0.376	14.6		

Table II also shows the evaluation of 30 simultaneous measurements of venous and finger-tip blood, obtained from 27 persons (14 males, 13 females), 25 of them healthy ones. The difference of the mean values of –0.25 Gm. is by far too small to be statistically significant. This would be the case only if it were more than twice its standard error (Fisher[9]). Ten of the 30 cases gave identical values; in 5 cases the finger-tip values were higher; in 15 cases they were lower than the venous ones.

TABLE III

STATISTICAL EVALUATION OF 40 COMPARISONS OF VENOUS AND EAR LOBE BLOOD

SOURCE	MEAN VALUE	STANDARD DEVIATION	STANDARD ERROR	COEFFICIENT VARIATION	DIFFERENCE OF MEANS	STANDARD ERROR OF DIFFERENCE
Venous	14.03	1.96	0.310	14.3	+1.35	0.46
Ear lobe	15.38	2.14	0.338	13.9		

Table III shows the evaluation of 40 simultaneous measurements of venous and ear lobe blood. These were carried out on 34 persons (12 males, 22 females), 4 of them suffering from anemia. The difference of mean values, +1.35 Gm., is nearly three times as great as its standard error and must, therefore, be considered statistically significant. In 3 cases both values were identical; in one case the ear lobe contained less, in 36 cases, it contained more hemoglobin than the arm vein. On the average, the ear lobe values are 10 per cent higher than the venous ones.

It should be emphasized that the manner in which the blood was taken from the ear lobe seemed to be without influence, as could be observed in separate experiments. The same high values were obtained whether a deep puncture wound with consequent free flow was made, or whether the blood was obtained by repeated squeezing. No change in the picture was seen when the ear blood was taken first, and the venous sample after a few minutes interval. Seasonal influences seemed likewise without effect. More than half of the trials were carried out during the winter and spring season, whose climatic conditions do not show very marked difference from those of American and European countries of the moderate zone.

COMMENT

Differences between venous and peripheral blood under standard conditions have so far been reported only in the case of pernicious anemia by Duke and Stofer,[10] who found higher values in the ear lobe. During the first days or weeks of life higher hemoglobin, red blood cell, and cell volume values were likewise observed by several authors in peripheral blood from the heel (e.g., Andresen and Mugrage[11]). The notorious high peripheral values in shock, caused by blood concentration, need scarcely be mentioned here.

The fact that such differences also exist in the healthy (and anemic!) adult in the case of the ear lobe should discourage the practice of taking blood from this source for hematologic diagnosis, a practice which is still used by a considerable number of physicians throughout the world. It further offers a possible explanation for the fact that the hemoglobin values in Williamson's[8]

classic investigation are uniformly about 10 per cent higher than those obtained by the majority of all other workers. Williamson had been working with blood from the ear lobe.

It seems conceivable that stasis in the capillaries and subsequent changes in the blood composition may more easily occur in the ear lobe than in the finger pulp. For a better understanding of the phenomenon, however, it is imperative to add red blood cell and hematocrit determinations to the hemoglobin values reported in the present paper. Work on this line is now in progress, and it can already be stated that a tendency for macrocytosis prevails in the ear lobe blood. It would be worth while to investigate if this also applies to body blood deposits.

SUMMARY

Venous blood has been compared under standard conditions with peripheral blood from the finger tip and the ear lobe.

No significant differences exist between the hemoglobin content of venous and finger-tip blood. Blood from the ear lobe, however, contains significantly more hemoglobin and should, therefore, not be used for diagnostic purposes.

I wish to express my gratitude to Dr. I. L. Fisher for his helpful advice and criticism throughout this study.

REFERENCES

1. Bürker, K.: Vergleichende Untersuchungen über den Gehalt des menschlichen Blutes von Hämoglobin und Erythrocyten in verschiedenen Teilen des Gefäss-Systems, Pflüger's Arch. f. d. ges. Physiol. **167**: 143, 1917.
2. Yarbrough, N.: Blood Counts With Oxalated Blood, J. Lab. & Clin. Med. **7**: 172, 1921.
3. Foord, A. G.: Blood Counts With Oxalated Blood Compared With Ordinary Counts, J. Lab. & Clin. Med. **8**: 343, 1923.
4. Reichel, J., and Monastero, G.: Beiträge zur Frage der numerischen Verteilung der Erythrocyten und Leukocyten an der Peripherie, Klin. Wchnschr. **8**: 1712, 1929.
5. Price-Jones, C., Vaughan, J. M., and Goddard, H. M.: Haematological Standards of Healthy Persons, J. Path. & Bact. **40**: 503, 1935.
6. Andresen, M. I.: A Method for the Collection of Peripheral Blood Samples, J. Lab. & Clin. Med. **23**: 751, 1938.
7. Heilmeyer, L.: Medizinische Spectrophotometrie, Jena, 1933, G. Fischer, p. 86.
8. Williamson, C. S.: Influence of Age and Sex on Hemoglobin, Arch. Int. Med. **18**: 505, 1916.
9. Fisher, R. A.: Statistical Methods for Research Workers, Edinburgh, 1936, Oliver & Boyd.
10. Duke, W. W., and Stofer, D. D.: A Comparison of Capillary and Venous Blood in Pernicious Anemia, Arch. Int. Med. **30**: 94, 1922.
11. Andresen, M. I., and Mugrage, E. R.: Venous and Peripheral Red Blood Cell Values, Am. J. Clin. Path. **8**: 46, 1938.

THE MEASUREMENT OF INTRAVENOUS BLOOD SUGAR CURVES*

BENGT HAMILTON, M.D., AND A. F. STEIN, M.D.
CHICAGO, ILL.

THE blood sugar curve, obtained after the administration of glucose, is a clinical laboratory test frequently employed, but only rarely giving any clear-cut information, except in cases of markedly abnormal carbohydrate metabolism. Although it is well known that there are great normal variations in blood sugar curves, variations noted when examining a number of persons, as well as one and the same person on different occasions, there is no simple way of describing these variations.

The following report describes a method by which the intravenous blood sugar curve may be characterized by a single figure. The method is based on the fact that, although a curve is difficult to describe, it is easy to measure the slope of a straight line. Many curves may be considerably straightened out by plotting on logarithmic paper; often this is true of the intravenous blood sugar curve to a very surprising degree, especially in the middle part of the curve, where the values fall in the range of normal postabsorptive values (from about 200 to 100 mg. per 100 c.c.). This is, probably, the most important part of the curve. In the higher ranges, never reached under normal conditions, it is possible that special mechanisms come into play (for instance, excretion of sugar through the kidneys). Toward the end of the curve, at values of about 100 mg. per 100 c.c., we might expect various mechanisms for stabilizing the blood sugar at normal values to become active. In the middle range, however, we would observe and study the normal mechanism for removal of excess carbohydrate from the blood after ingestion of carbohydrate, and it is interesting that it is in this range that the logarithmic curve so often forms a straight line.

METHOD

The blood sugar values are plotted on semilogarithmic graph paper, and the part which forms an approximately straight line is noted. In questionable cases, where it is difficult to decide how much of the curve is approximately straight, the decision is not of very great importance, as long as the selected part includes most of the values between 200 and 100 mg. per 100 c.c. As will be shown later, including one value more or less will not greatly change the final result. The logarithms of the blood sugar values forming the end points of the selected part of the curve are then obtained, with two decimals, from a table or slide

*From the Department of Pediatrics, University of Illinois College of Medicine, and the Children's Division of Cook County Hospital, Chicago.
This work was supported by a grant from Mead Johnson & Company, Evansville, Ind.
Received for publication, August 4, 1941.

rule. The difference between these two logarithms, divided by the time elapsed between these two points of the curve, is an approximate measure of the slope of the curve.

TABLE I

MIN. AFTER INJECTION	BLOOD SUGAR	LOG BLOOD SUGAR	CHANGE IN BLOOD SUGAR
2	280	2.45	
15	220		60
30	161		51
45	124		37
60	99	2.00	25
75	101		+2
90	98		3

Table I gives the blood sugar values obtained in Case 2 (third from the left in the second row in Fig. 1). An approximately straight line is formed between the values 280 (first value) and 99 (fifth value). The difference between the logarithms of these two values is 2.45 – 2.00 = 0.45. The value 280 was obtained two minutes after the injection of glucose; the value 99, sixty minutes after the injection; the elapsed time is, therefore, 58 minutes. In this period of time the logarithm of the blood sugar decreased 0.45. In one minute, then, it decreased on the average 0.00776. This value, 0.00776 per minute, is a measurement of the slope of the logarithmic curve. However, a decrease of the logarithm of the blood sugar of 0.00776 per minute is the same as if the actual blood sugar value had been divided by 1.0181 each minute. Dividing by 1.0181 is the same as multiplying by 0.9823. In other words, the blood sugar at the end of each minute is in this case on the average 98.23 per cent of what it was at the beginning of the minute, which is a decrease of about 1.77 per cent. This figure, 1.77 per cent per minute, is the measure of the rate of decrease of sugar in the blood in this case. Fortunately, this rather complicated calculation made to arrive at this figure is not necessary. If the slope of the logarithmic curve (in this case 0.00776) is multiplied by 230, we will always arrive at approximately the same result as that obtained in the above calculation.* In this case, we get 1.78 per cent per minute by using this method, while the more elaborate calculation resulted in the value 1.77 per cent per minute. As the accuracy of the method justifies the inclusion of only one decimal, the difference is not important. For practical purposes it is sufficient to say that the rate of decrease of blood sugar in Case 2 was 1.8 per cent per minute.

It must be very strongly emphasized that this figure, 1.8 per cent per minute, is not the same as 18 per cent in ten minutes, or 180 per cent in one hundred minutes; that is, it is not the same if percentage change is calculated, as is usually done, in per cent of the initial figure at the beginning of the period of change. If, however, the percentage change is calculated in per cent of the geometric mean between the initial and the final figure during the period of change, then it will be approximately correct to multiply per cent per minute by any number of minutes.

*This statement is based on the fact that $\frac{d}{dt}(\log y) = \frac{1}{y} \cdot \frac{dy}{dt}$.

The multiplication by 100 is made to change a fraction into percentage; by 2.30, to change ordinary into natural logarithms.

Summary of Method.—Plot the blood sugar curve on semilogarithmic paper (obtainable in most stationery stores). Determine which part of the curve is approximately straight. Obtain from a table or slide rule the logarithms (with two decimals) of the two figures forming the beginning and end of the straight part of the curve. Take the difference between these two logarithms, divide this difference by the number of minutes elapsed between the two blood sugar determinations, and multiply the result by 230. The figure obtained is the average rate of decrease of the blood sugar in per cent per minute.

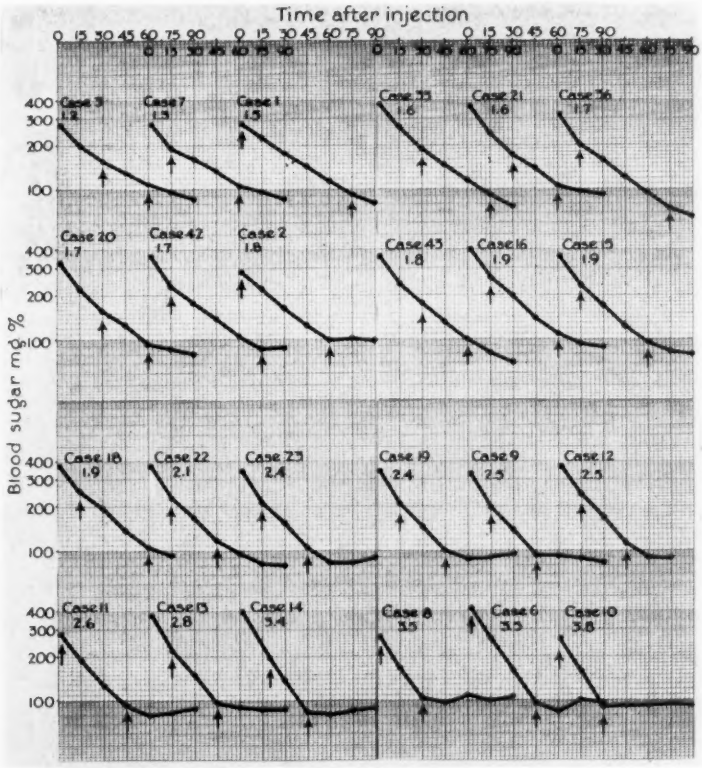


Fig. 1.—Intravenous blood sugar curves from approximately normal persons plotted on logarithmic paper. The part of the curve used for estimating the slope is marked by arrows. The figures indicate the slope expressed in per cent per minute decrease.

We have made intravenous blood sugar curves on a number of patients with miscellaneous disorders, all children; old burns, kept in the hospital for grafting; patients with gonorrheal vaginitis; fractures; patients with bronchiectasis. Some of these have received a diet high in carbohydrate, others a diet low in carbohydrate; many were tested on both diets. We shall not give the data as to the composition of the diet, since the lack of a metabolism ward made it quite certain that the diets were only approximately followed. The diets as offered had a ketogenic-antiketogenic ratio of about 0.25:1 for the high carbohydrate diet, 2:1 for the low carbohydrate diet. All we can definitely say is that these two diets were very different in their carbohydrate content.

The technique of the intravenous test is important for the interpretation of the curve. It goes without saying that the amounts of sugar given should be

strictly standardized. In our tests we have given 0.5 Gm. of glucose per kilogram of body weight in a 20 per cent solution (in normal saline). The amounts of dextrose injected have a marked effect on the curve.[1] In some cases we have given smaller amounts and found that on these smaller amounts the curves tend to be irregular and difficult to interpret. The time used for injection should be made as uniform as possible by using the same size intravenous needle and the same pressure in every case. We have used a No. 18 intravenous needle and a salvarsan tube with very rapid drip at a distance of six feet from the floor. In this manner, the injection was usually completed in less than one minute. According to Tunbridge and Allibone,[1] small differences of two to four minutes in the time used for injection are of no importance for the result.

RESULTS

In Fig. 1 are plotted on logarithmic paper our 24 patients, more or less normal, on a high carbohydrate diet. The curves are ranged according to the steepness of the slope, and the figures above the curves indicate the steepness in terms of per cent decrease per minute. The part of each curve chosen for the calculation of the slope is indicated by the arrows. It may seem as if this part, in some cases, was rather arbitrarily chosen, but as has already been pointed out, it makes very little difference in the result of the calculation if one only adheres to the principle of (1) choosing as straight a part of the curve as possible, and (2) in questionable cases to include approximately the range from 200 to 100 mg. per cent. In Case 20, for instance, it may seem questionable whether to calculate from the second or the third value; calculating from the second, one obtains a slope of 1.9 per cent per minute; calculating from the third, the value obtained is 1.7 per cent per minute. In Case 3, calculating from the second value the slope is 1.4, from the third value, it is 1.2 per cent per minute. Going over a number of curves in this manner we have found that the error of the method is, approximately, 0.2 per cent, as far as the calculation of the slope is concerned. It seems likely that if we assume a total error of twice as much, ±0.2 per cent, we shall not overestimate the accuracy of the method. That would mean that values more than 0.4 per cent apart should be considered as showing a real difference. The total range of our values is from 0.9 to 3.8 per cent per minute (a difference of 2.9 between the highest and the lowest value), and with an error of 0.4 per cent we would, then, be justified in recognizing seven different degrees of steepness. This, it seems to us, is a worth-while refinement on the usual method of designating the curves as "steep," "normal," or "flat."

In Table II may be seen the distribution of the values for the slope of the curve (the removal rate of sugar from the blood) in patients on a high and on a low carbohydrate diet. (Four more curves are included than in Fig. 1, the additional four being repeated curves on cases previously tested.) The table shows how on the high carbohydrate diet the most common removal rate was between 1.5 and 1.9 per cent per minute (10 cases), while on the low carbohydrate diet the most common rate was between 1.0 and 1.4 per cent per minute. A very interesting fact stands out: There was considerably more variation

between the cases on a high carbohydrate diet than between those receiving a low carbohydrate intake (16 cases were tested on both diets; the difference in variability may, therefore, hardly be ascribed to a difference in case material). This is all the more striking, since we feel sure that the low carbohydrate diet was more often broken than the high carbohydrate diet. The findings seem to indicate that when the carbohydrate metabolism is understimulated (low carbohydrate diet), the reaction to a sudden demand on this metabolism (intravenous injection of glucose) is very much the same in all persons; while there seems to be considerable individual variation in the response when the metabolism has previously been stimulated by a high carbohydrate intake. We may also conclude that if we plan to study individual variations in carbohydrate metabolism, it seems more likely that a high carbohydrate intake will bring out such variations than a low carbohydrate diet. In some persons, tested on both diets, the difference in response was very marked; in others the difference was small. The difference in response was nearly always due to differences in the response to the high carbohydrate diet, since the removal rate on the low carbohydrate diet was very much the same in all cases.

TABLE II

DISTRIBUTION OF PER CENT PER MINUTE DECREASE OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION

CARBOHYDRATE DIET	NUMBER OF CASES					
	LESS THAN 1.0	1.0-1.4	1.5-1.9	2.0-2.4	2.5-2.9	3.0+
High	-	4	10	5	5	4
Low	1	17	7	-	-	-

TABLE III

CASE NO.	REMOVAL RATE AFTER 3 DAYS ON HIGH CARBOHYDRATE DIET; % PER MIN.	REMOVAL RATE AFTER DIET CHANGED TO LOW CARBOHYDRATE	
		AFTER 2 DAYS	AFTER 5 DAYS
44	1.7	1.0	1.0
45	2.5	1.4*	1.0
46	1.6	1.0	1.2
47	2.5	1.8	1.2

*Technique not quite good.

It is, of course, a well-known fact that the preceding diet is of great importance for the outcome of the blood sugar curve. According to Tunbridge and Allibone,[1] the patient should be on the diet two weeks before the test is made, a condition hardly possible to fulfill in clinical practice. To judge from our findings, the diet is of more importance in some persons than in others. Not only, as mentioned above, that some persons respond very strongly to a high carbohydrate diet (steep curves) while in others the response is much less marked; but it seems also that the maximum effect of a change in diet is obtained sooner in some persons than in others. In Table III may be seen the result obtained by suddenly changing the diet in four cases. In those two (Case 44 and Case 46) where the removal rate was about average on a high carbohydrate diet, no more effect of the change was seen after five days than

was already present after two days. In the two remaining cases, however, the removal rate was rather rapid on the high carbohydrate diet, and in these two cases the effect of this diet seemed to last longer. If the values obtained after only two days on a low carbohydrate diet had been accepted as final, one would, obviously, have obtained an erroneous impression as to the speed of removal on this diet. For this reason, we have included in our table only the cases where the diet had been given at least four days previous to the test.

COMMENT

A simple way of measuring the intravenous curve is that used by Crawford,[2] who classifies the curve according to the time it takes after the injection for the blood to reach normal levels. We are of the opinion that the intravenous blood sugar curve deserves a somewhat more accurate method of measuring than is obtained in that manner. For instance, measured according to Crawford, the five first curves in Fig. 1 would be all the same, while a brief look shows that the fifth (Case 21) is steeper than the first (Case 3). The method also would indicate differences where, in our opinion, none exist, as for instance between the fifth case in the first row (Case 21) and the sixth case (Case 36). The former (Case 21) reached the level of 100 mg. in about sixty-seven minutes; the latter (Case 36), in about fifty-five minutes. According to our calculation, the curves are about alike and the difference obtained by the other method of estimation is due entirely to the fact that the curve of Case 21 starts at a higher level than that of Case 36. Compare also the two last curves in the diagram, Cases 6 and 10. Here again, the curves would be very different according to Crawford, but about the same according to our method of measuring. It seems questionable to us whether the absolute height to which the curve rises—which plays such an important role in the method of Crawford—is not much less important than the steepness of the slope, which is the only characteristic measured by our method. In any case, the steepness should be measured; then, as a second characteristic, may, perhaps, be added the maximum rise.

Ross and Tonks[3] are, in our opinion, using a somewhat too complicated method for measuring the intravenous curves. These authors measure the surface area under the curve by means of a planimeter. In addition to this being a somewhat elaborate method, it also seems to us that from a mathematical standpoint the differential of the blood sugar curve, which is determined by our method, has more meaning than the integral, determined by Ross and Tonks.

Although the purpose of our study was to find a practical method of measuring variations in the intravenous blood sugar curve, a fact of more theoretical interest became apparent from our data: Whenever the intravenous curve forms a straight logarithmic line, the rate at which the sugar is being removed from the blood at a given moment is directly related to the amount of sugar present in the blood at that moment; being always the same percentage of the amount of sugar present.

Another interesting fact is that in at least ten of our curves an approximately straight line slope changes suddenly into a horizontal line (L-shaped curves). This indicates that at a given level of blood sugar the removal of sugar from the blood suddenly stops, or, which is more probable, at a given

level sugar begins to enter the blood at the same rate as it is removed. If the latter is true, we would, then, have a method of calculating the rate at which—in a given individual—the sugar enters the blood at a given time; in other words, the rate of flow of sugar from stores and alimentary tract. For instance, if a curve with a slope of 2 per cent per minute suddenly becomes horizontal at the level of 100 mg. per cent, we might assume that this equilibrium is accomplished by an inflow of 2 per cent per minute, or approximately 2 mg. for every 100 c.c. of blood. If such a person had 10 liters of blood, he would have to consume, on the average, 12 Gm. of sugar per hour to maintain the rate of supply of sugar to the blood. It may thus be seen that the assumption we have made leads to rather reasonable figures for daily carbohydrate intake.

SUMMARY

A method is described by which the slope of the intravenous blood sugar curve may be calculated in a simple manner and described by a single figure.

By using this method, it was shown that the slope of the intravenous curves was surprisingly constant when the patients received a low carbohydrate diet; on a high carbohydrate diet the patients showed considerable individual variations.

REFERENCES

1. Tunbridge, R. E., and Allibone, E. C.: The Intravenous Dextrose Tolerance Test, Quart. J. Med. (New Series) **9**: 11, 1940.
2. Crawford, Theodore: A Standard Intravenous Glucose Tolerance Test, Arch. Dis. Child. **13**: 69, 1938.
3. Ross, C. Wallace, and Tonks, Eva L.: The Determination of Glucose Tolerance, Arch. Dis. Child. **13**: 69, 1938.

THE USE OF DIHYDROTACHYSTEROL IN PARATHYROPRIVIC TETANY*

Report of a Case

Floyd E. Harding, M.D., Los Angeles, Calif.

THE occurrence of tetany following removal of the thyroid gland with destruction or loss of the parathyroids is unusual, owing to the improvement in surgical technique that has taken place. There are normally four parathyroid glands, two on each side of the thyroid, but their number and location vary considerably due to defects in embryologic development. Because of this anomaly and their small size, they are often partly removed. Hypertrophy of the remaining glandular tissue usually brings about a cure in a few months. In rare instances so much gland tissue has been removed that tetany becomes a permanent condition. The case reported here is of this type, since regeneration did not occur after one and a half years, and the treatment presented an ideal clinical subject for observation of the effect of a new drug.

*From the Department of Medicine, Ross-Loos Medical Group, Los Angeles.
Received for publication, November 25, 1940.

Dihydrotachysterol is made from tachysterol, a by-product of the irradiation of ergosterol with ultraviolet light. It will raise the blood calcium to normal or even above normal when given by mouth, and for this reason, has an advantage over parathyroid extract, which is given hypodermically. The effect of parathyroid extract is of short duration, not over forty-eight hours. When dihydrotachysterol is used, the blood calcium does not begin to rise until after forty-eight hours, reaches a maximum in about one week, and decreases in one to three weeks after its use is discontinued. When too much dihydrotachysterol is used, the patient complains of asthenia, nausea, vomiting, headache, stupor, thirst, or skin rash. The method of management of a patient under this treatment, with frequent check on the blood chemistry or urine, is described by Albright.[2] The urine test can easily be performed by the patient at home. Sulkowitch reagent is added to urine, and the observation of a fine, white precipitate with a normal excretion of calcium is indicative of a normal blood calcium. If the solution remains clear, hypocalcemia is present. In hypercalcemia the solution becomes milky with increased output of calcium.

Hypoparathyroidism causes low blood calcium, high blood phosphorus, decreased excretion of calcium in the urine, and positive calcium balance (less excretion than intake). What becomes of the calcium that is ingested is a mystery, since density of the bones is not evident on radiologic examination. It has been suggested that the calcium is deposited in the tissues. As the blood calcium goes down, the phosphorus goes up, and vice versa, so that it is necessary to decrease the blood phosphorus by a diet low in phosphorus in order to facilitate raising the calcium. While dihydrotachysterol raises the blood calcium, it does not mobilize the calcium in the blood by removing it from the bones, as is the case when parathyroid extract is used. Dihydrotachysterol will not prevent or cure rickets as irradiated ergosterol does, so that the latter is necessary to improve bone quality. It is better, therefore, to combine vitamin D, calcium, and dihydrotachysterol in the treatment of parathyroprivic tetany.

The dosage of dihydrotachysterol is not well established. It varies with the amount of the gland removed and the degree of regeneration. If all parathyroid tissue has been removed, the dosage is more constant. Adams[1] reported a case of short duration following subtotal thyroidectomy in which the patient was symptom free on 1 c.c. twice a week. Newman[5] reported a case of parathyroprivic disease with tetany of seven years' duration in which 1 c.c. of dihydrotachysterol daily was necessary in spite of the fact that the blood calcium ranged from 8 mg. to 12.6 mg. for each 100 c.c. of blood before treatment. The use of the drug in idiopathic tetany[3, 4] and scleroderma[6] is somewhat different from that following accidental removal of the parathyroids.

The case presented here is unusual and interesting for the following reasons: (1) absence of parathyroid tissue and the appearance of tetany any time treatment is discontinued; (2) dosage of dihydrotachysterol; (3) persistence of considerable laryngeal stridor even with a blood calcium of 9 mg.; (4) further improvement of laryngeal stridor with thyroid extract; (5) decalcification of jawbone; and (6) injury to the recurrent laryngeal nerve.

REPORT OF CASE

The patient, a schoolteacher, aged 46 years, was referred for treatment on August 26, 1939. She complained of cramps in her arms and hands. During the spasms she could not straighten out her hands and fingers. There was extreme weakness with the attacks which were relieved only by an injection of parathyroid extract about every other day. She talked mainly in a whisper, but occasionally made rather high-pitched noises. Nervousness and irritability were pronounced. Her ankles were slightly swollen.

She gave a history of thyroidectomy in July, 1928. In June, 1939, a large adenoma was removed from the region of the right upper part of the thyroid, in which was imbedded the right upper parathyroid. The tetany first appeared following the latter operation. Other operations included tonsillectomy, appendectomy, uterine suspension, and a few dental extractions.

She stated that the goiter had originally appeared during puberty. Following thyroidectomy she felt well until 1932, when she had a nervous breakdown. The history from 1932 to 1939 was supplied by several doctors who had treated her during that period. The following basal metabolism readings were recorded during these years: 1932, +31, +24, +10, +15, +28, +30; 1933, +26, +26; 1934, +25; 1935, +14, +44, +35; 1936, +31, +39; 1937, +19, +19, +24, +28; 1938, +30; and 1939, +35, +44. The Wassermann test, urinalysis, and blood count were normal in 1934. A small mass became evident in the region of the upper half of the right lobe of the thyroid, and continued to increase in size. The patient presented symptoms of exhaustion, nervousness, pounding of the heart, protrusion of the eyeballs, loss of weight, and slight tremor of the fingers. Her pulse rate varied from 100 to 140; her blood pressure was 130 systolic and 75 diastolic. The menstruation was regular every twenty-eight days. The diagnosis was made of chronic nervous exhaustion, with adenoma of the right lobe of the thyroid and mild hyperthyroidism. Removal of the adenoma was advised by several doctors, but the patient preferred medical treatment and was given Lugol's solution, sedatives, and other treatment at various times until she submitted to removal of the adenoma.

Physical examination on the first visit here was essentially normal. The scar on the neck was slightly tender. The pulse rate was 84. Medication for each day consisted of one vitamin ABDG capsule, 6 tablets of calcium gluconate of 15 grains each, 6 tablets of desiccated parathyroid of 0.1 grain each, and 60,000 U.S.P. units of irradiated ergosterol. She was receiving parathyroid injections about every other day.

In September, 1939, dihydrotachysterol, 1 c.c. every three days, was added to the treatment and later increased to 1 c.c. daily, since the blood calcium was still 7.5 mg. for each 100 c.c. of blood. The patient was started on a high-calcium, low-phosphorus diet. The parathyroid injections were decreased to two a week and later to one a week. She began feeling stronger and gaining weight and was advised to start walking for exercise. Her basal metabolism was +12, her urinalysis was negative, and her blood pressure was 88 systolic and 60 diastolic.

In October she was much better, and showed some improvement in the voice and no swelling of the ankles. Since she was markedly nervous it was still necessary for her to rest all the time. With exercise the muscles between the shoulders would cramp, but there was no definite tetany; she had discontinued the parathyroid injections and tablets, as well as the irradiated ergosterol. At night during sleep she made sounds resembling snoring that kept her husband awake. One-half grain of phenobarbital at bedtime was prescribed. Her blood pressure was 98 systolic and 68 diastolic, her blood calcium was 9 mg., and her basal metabolism rate averaged –9.

In November and December she showed general improvement, but as her voice improved the breathing noises at night increased. She tried reducing the dihydrotachysterol from 15 minims to 10 minims daily, but her condition became worse and she had to resume taking 1 c.c. daily. Daily urine tests with the Sulkowitch reagent were started, and there was a fine, white precipitate most of the time, although occasionally the urine was clear or milky. Her blood calcium was 8 mg., her basal metabolic rates were –10 and –25; red blood cells 4,270,000, hemoglobin 74 per cent, and the urinalysis negative. Her weight increased to 147 pounds. The hair on her legs, head, and eyebrows, which had turned white, began to get darker. One-fourth grain of thyroid was added to the treatment.

In January and February, 1940, she showed general sluggishness, with some cramps in the legs and some shortness of breath. Her blood pressure was 100 systolic and 68 diastolic, her blood calcium was 8.5 mg., her pulse rate was 70 to 86, she weighed 154½ pounds. The urinalysis was negative. The thyroid extract was increased to 0.5 grain daily. She discontinued taking vitamin D. By March her voice had improved considerably. It was felt that the recurrent laryngeal nerve had regenerated, but there was now a loud crowing noise on inspiration and shortness of breath on climbing stairs. She stopped taking the thyroid extract, since she thought that she felt better without it. An electrocardiogram showed marked myocardial damage. Urinalysis was normal. She was advised to continue resting and to eat more fruits and vegetables and less of fattening foods. A postcard arrived in April, 1940, stating that she had stopped the dihydrotachysterol for ten days, but had a bad attack of tetany and had to take an injection of parathyroid. She had severe cramps, and could not straighten out her hands. The dihydrotachysterol was resumed.

The patient was not heard from again until July. The drops had been discontinued for three weeks. The tetany with carpopedal spasm had reappeared, and she had difficulty in breathing with loud tracheal stridor. A few injections of parathyroid helped to relieve the cramps, but there was considerable weakness and nervousness. She was placed in the hospital where she remained for two weeks. She made an uneventful recovery on 1 c.c. of dihydrotachysterol daily. Examinations by a surgeon, otolaryngologist, and neurologist were all essentially normal. The vocal cords were functioning normally, and all agreed with the diagnosis of tetany and laryngeal stridor due to parathyroprivia. The eyes were tested and glasses were fitted to correct a slight visual disturbance.

On the day the dihydrotachysterol was resumed, the blood calcium was 5.3 mg.; a few days later it was 7.2 mg., with 6.5 mg. of phosphorus. The hemoglobin was 69 per cent, red blood cells 4,380,000, color index 0.8, white blood cells 9,000, platelets and differential cells normal. The temperature ranged between 97.4° and 99° F., usually slightly subnormal. In August the calcium tablets were discontinued, and two teaspoonfuls of powdered calcium gluconate, three times daily, were given in their place. Marked laryngospasm and tracheal stridor continued in spite of this, although both symptoms improved to some extent. The urine tested with Sulkowitch reagent was always milky. Dental x-ray examination showed some decalcification of the jawbones. A roentgenogram of the spine showed no general decalcification. The blood pressure was 110 systolic and 72 diastolic. The basal metabolic rate was –27.

In September the patient was much better; she looked rested and felt stronger, but she still had dyspnea with loud inspiration noises. When she was asleep, her breathing could be heard all over the house. Menstruation was still regular every month. Foods containing phosphorus were eliminated from the diet. Irradiated ergosterol, 3,500 U.S.P. units, was given after each meal. Blood calcium was 8.5 mg. At the beginning of the second week in September, 1 grain of desiccated thyroid was given daily. Blood calcium was 7 mg., and blood phosphorus was 4.1 mg. The pulse rate was 80, and the blood pressure was 100 systolic and 72 diastolic.

On Monday, September 16, 1940, the patient was feeling much better and started to work. By September 20 she was feeling stronger, although she had finished one week of work. She was breathing easier and with much less noise. Her pulse rate was 80. On September 27 she was feeling very well; she had no dyspnea and no stridor at all when sitting, and only slight musical noise on inspiration during exercise. At the end of the second week of work she felt she had succeeded in her effort to get back on the job. This was the first time she had worked since June, 1939. During October she still continued in relatively good health. Her blood calcium was 8.3 mg. and her blood phosphorus was 5.3 mg.

DISCUSSION

In the case just presented, the laryngeal stridor was the most difficult symptom to overcome. It was necessary to give intensive treatment with a combination of drugs and diet to improve the calcium metabolism before improvement occurred. The dihydrotachysterol relieved the irritability and spasm

of the muscles, and the thyroid improved the muscle tone and use. These two drugs were the most useful in the treatment of the case. It was necessary to keep a close check on the diet. The dosage of all medication essential to create and to maintain a state of good health was larger than expected. The treatment was expensive, since the monthly allowance of 30 c.c. of dihydrotachysterol plus the other drugs averaged about $20.00.

A milky urine when tested with Sulkowitch reagent did not mean that hypercalcemia was present. When larger doses of calcium were given by mouth to this patient, the urine test was always milky, although the blood calcium was only 8 or 9 mg. for each 100 c.c. of blood. The patient is going to try to take less of the calcium gluconate with the same amount of the other drugs since she is probably taking more calcium than is necessary for maximum improvement. Of course, there is no danger of a high blood calcium if the urine is clear or slightly cloudy. The test is of diagnostic value before treatment, and it can be used during treatment if smaller amounts of calcium are given. When the urine test is milky, a blood test is necessary to determine the exact level of the blood calcium.

SUMMARY

The treatment of a case of parathyroprivic tetany with injury to the recurrent laryngeal nerve is presented. The recurrent laryngeal nerve regenerated in a few months, but the deficiency in parathyroid hormone continued after one and one-half years. Maximum improvement was brought about only after intensive treatment with a low phosphorus diet, calcium gluconate, irradiated ergosterol, dihydrotachysterol, and thyroid extract. Dihydrotachysterol raised the blood calcium, prevented the tetany, and partially decreased the laryngeal stridor. The thyroid extract raised the basal metabolism and further improved the laryngeal stridor. The minimum dosage of dihydrotachysterol was 1 c.c. daily. There was decalcification of the jawbones, but no general decalcification of the skeleton.

REFERENCES

1. Adams, L. J.: Post-operative Parathyroid Tetany Treated With Dihydrotachysterol (A-T-10), Canad. M. A. J. **42**: 373, 1940.
2. Albright, Fuller: Note on the Management of Hypoparathyroidism With Dihydrotachysterol, J. A. M. A. **112**: 2592, 1939.
3. Berk, J. E.: Clinical Experience With Dihydrotachysterol in the Management of Idiopathic Hypocalcemia, Endocrinology **25**: 984, 1939.
4. Franco, Saverio C.: Parathyroid Tetany: The Chronic Idiopathic Parathyroid Insufficiency of 10 Years' Duration Successfully Controlled With Dihydrotachysterol, Ann. Int. Med. **14**: 529, 1940.
5. Newman, H. F.: Case of Hypo-parathyroidism Treated With Dihydrotachysterol, J. Mt. Sinai Hosp. **6**: 327, 1940.
6. Perez, W. M., Soffer, L. J., and Silbert, S.: Improvement in 2 Cases of Diffuse Scleroderma by Use of Dihydrotachysterol (A-T-10); Preliminary Report, J. Mt. Sinai Hosp. **6**: 333, 1940.

THE COMPARATIVE PHYSIOLOGIC VALUE OF INJECTED CAROTENE AND VITAMIN A*

JANE G. LEASE, PH.D., E. J. LEASE, PH.D., H. STEENBOCK, PH.D., AND C. A. BAUMANN, PH.D., MADISON, WIS.

MANY workers have observed that carotene or vitamin A may be biologically active when injected into experimental animals (Wollman and Vogliano;[1] Koehne and Mendel;[2] Wolff, Overhoff, and van Eckelen;[3] von Euler and Rydbom;[4] Rydbom;[5] Gordon and Titherington;[6] Chu and Coady;[7] Lasch;[8] Greaves and Schmidt;[9, 10] Guilbert and Hart;[11] Wilson, Ahmad, and Majumdar;[12] Phillips and Bohstedt[13]). Parenteral carotene and vitamin A have also been reported to be effective in human therapeutics (Blegvad;[14] Edmund;[15] Bloch;[16, 17] Buschke;[18] Josephson and Freiberger[19]). However, there is also abundant evidence that injected vitamin A is not always efficiently utilized. Funk[20] stated that vitamin A given parenterally was without any activity. Wollman and Vogliano[1] observed that repeated injections of cod-liver oil or butter oil were not tolerated well, since no growth resulted until after injections were stopped.

Koehne and Mendel[2] observed only temporary remission of deficiency symptoms with the accumulation of much foreign fat at the site of injection when butter oil or cod-liver oil were injected into depleted rats. Von Euler and Rydbom[4] found that 0.050 mg. of carotene injected daily was inadequate for growth in depleted rats, although much smaller amounts given orally were known to restore growth. Chu and Coady[7] also reported better results with carotene given orally than with that given parenterally. Drummond and coworkers[21-24] failed to find any storage of vitamin A in the livers of rats and cats when carotene, emulsified in glucose solution, was injected intravenously. In one experiment with rabbits, however, some vitamin A was stored. This relative inertness of parenteral carotene has also been observed by Ahmad, Grewal, and Malik[25] in their work with rats and dogs.

Furthermore, preliminary experiments of our own indicated that injected carotene was not converted into vitamin A efficiently. Rats with stores of 175 blue units per liver were fed a fat-free diet, and then given intraperitoneal injections of large amounts of an aqueous suspension of colloidal carotene. No vitamin A could be detected in the livers after five weeks, although much carotene was found at the site of injection, adherent to the liver.

Parenteral administration of vitamin A sometimes appears to be indicated in human therapy (Blegvad;[14] Bloch;[16, 17] Gordon and Titherington;[6] Edmund

*Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Madison.

Received for publication, December 4, 1940.

and Clemesen;[26] Finkelstone, Howard, and Paris;[27] Decourt;[28] Buschke;[18] Josephson and Freiberger[19]). However, experimental data on the relative efficiencies of the various means of administration are lacking even with animals. We have, therefore, carried out experiments with rats, using several biological criteria, such as cure of ophthalmia, duration of cure, and growth and survival time, together with data on the carotene and vitamin A content of the tissues.

METHODS

Rats 3 weeks old and 35 to 50 Gm. in weight were fed a basal ration composed of casein (heated at 95° C. for fourteen days) 18 per cent, Osborne and Mendel salt mixture (as modified by Hawk and Oser[29]) 4 per cent, brewer's yeast 7.9 per cent, irradiated brewer's yeast 0.1 per cent, hydrogenated fat* 10 per cent, and sucrose 60 per cent. Food and water were given ad libitum throughout both the preliminary and experimental periods. Supplements of carotene or vitamin A were administered when the animals showed a loss in weight and the initial stages of ophthalmia. The supplements were given in the form of colloidal water suspensions or as oil solutions. Both were fed as well as injected. The amounts given ranged from 75 to 1,500 μg. The volumes of the doses did not exceed 0.25 c.c. for the oil solutions, and 1 c.c. for the colloidal suspensions. A detailed account of the various doses administered is given in the tables of results. In the evaluation of the biological response primary emphasis was placed upon the cure of ophthalmia because of the possibility that repeated injections might interfere with growth.[1] At the end of the experimental period the animals were killed and the tissues were analyzed for carotene and vitamin A.

The aqueous colloidal suspensions of carotene were prepared by slowly adding carotene in acetone solution to hot (90° C.) water agitated with a stream of nitrogen. The preparations were made fresh every three days and filtered to remove visible particles. A similar procedure was used for the preparation of aqueous colloidal solutions of vitamin A. The source of vitamin A was halibut-liver oil.† Its unsaponifiable matter was dissolved in methyl alcohol, chilled to −10° C., and filtered. The filtrate was dried under nitrogen and reduced pressure, then dissolved in acetone, and slowly added to hot (90° C.) water agitated with nitrogen.

For its quantitative determination the carotene was taken up in chloroform, and the intensity of absorption was measured at 485 and 460 mμ in a Bausch & Lomb spectrophotometer. Animal tissues were first dissolved in 8 per cent potassium hydroxide at room temperature overnight, and then 2 volumes of water with sufficient alcohol to make a concentration of 15 per cent alcohol were added. The carotene was extracted with peroxide-free ether, the extracts were washed with water and evaporated in vacuo, and the residue was dissolved in chloroform.

A similar procedure was employed in the preparation of extracts suitable for the determination of vitamin A by the colorimetric method of Carr and Price.[30]

*A mixture of partially hydrogenated vegetable fats marketed under the trade name "Spry."

†The halibut-liver oil was a commercial brand known as Haliver Oil, furnished by the Abbott Laboratories.

PARENTERAL ADMINISTRATION OF CAROTENE

(a) *Physiologic Requirements.*—When aqueous colloidal carotene was injected in amounts equivalent to 375, 750, or 1,500 μg per rat per week, ophthalmia was cured and growth was restored (Table I). Growth was equally good with injections of 750 or 1,500 μg a week, with an approximate gain of 60 Gm. in weight in five weeks. This was also the rate of growth observed when 75 μg of colloidal carotene were given weekly by stomach tube or when 7 μg of carotene were fed in oil solution by dropper. Thus, the amount of carotene necessary to produce a given biological response may vary by over a hundredfold, depending upon the mode of administration. Lesser amounts of carotene produced lesser gains in weight, e.g., the intraperitoneal injection of 375 μg of colloidal carotene per week resulted in a gain of only 37 Gm. in five weeks, whereas 75 μg per week failed to restore growth, to cure ophthalmia, or to maintain life. At our levels of administration (Table I) no difference in the response of animals injected subcutaneously or intraperitoneally was noted. Furthermore, carotene injected in oil appeared to be as effective as colloidal carotene. The presence of 5 per cent of glucose in the suspension of colloidal carotene failed to affect the results in any way.

TABLE I

EFFECT OF CAROTENE ADMINISTERED IN VARIOUS WAYS

LEVEL OF CAROTENE GIVEN (μg/WK.)	MEDIUM	METHOD OF ADMINISTRATION	AVERAGE GAIN IN WEIGHT AT 5 WK. (GM.)	AVERAGE CAROTENE IN LIVER AT DEATH (μg)	AVERAGE CAROTENE AT SITE OF INJECTION (μg)
1,500	Water	(a) Parenteral* Intraperitoneal	62	365 (211 to 507)	1,807 (1,605 to 1,990)
1,500	Water	Subcutaneous	63	106 (14 to 153)	1,860 (1,560 to 2,220)†
750	Water	Intraperitoneal	57	185 (112 to 246)	750 (585 to 1,030)
750	Water	Subcutaneous	62	129 (118 to 141)	543 (480 to 600)
750	Water (glucose)	Intraperitoneal	69	184 (126 to 274)	619 (576 to 658)
750	Oil	Intraperitoneal	67	45 (21 to 87)	1,766 (1,185 to 2,140)
375	Water	Intraperitoneal	37	44 (6 to 90)	40 (15 to 75)
75	Water	Intraperitoneal	All dead	5 (trace to 15)	4 (2 to 8)
75	Oil	Intraperitoneal	All dead	8 (trace to 11)	153 (115 to 229)
750	Water	(b) Oral Stomach tube	91	–	–
75	Water	Stomach tube	58	–	–
7	Oil	Dropper	60	–	–

*At least 3 animals were used at each level.
†Some carotene was also found in the mesentery and omentum.

The potency of colloidal carotene given orally was demonstrated not only by the amount of growth elicited, but also by the fact that 750 μg given each week for five weeks resulted in the hepatic storage of 567 blue units of vitamin A. By way of contrast the injection of 1,500 μg of colloidal carotene failed to yield any stores of vitamin A. Carotene injected in oil likewise failed to produce stores of vitamin A.

Injected carotene, however, accumulated in large amounts in tissues other than the liver. Both the amount and the location of these deposits depended upon the method of administration. After intraperitoneal injection, carotene was found in the omentum, mesentery, lymph nodes, and in the abdominal fat and

membranes surrounding the liver. After subcutaneous injection, carotene was found in similar areas and also at the site of injection (Table I). Oil solutions of carotene given intraperitoneally remained in the abdominal cavity for a considerable period of time, and much less carotene migrated to the liver than after the injection of the colloidal preparation (Table I). Apparently carotene was treated in part as a foreign substance because young rats injected with India ink showed a distribution of carbon particles similar to that of carotene. The presence of carotene at the site of injection has been reported previously (van den Bergh, Muller, and Broekmeyer;[31] Rydbom;[5] Rea and Drummond;[21] Wilson, Ahmad, and Majumdar[12]).

TABLE II

AVAILABILITY OF CAROTENE DEPOSITS FORMED BY PARENTERAL ADMINISTRATION

TOTAL CAROTENE GIVEN (μg)	MEDIA	METHOD OF ADMINISTRATION	DAYS TO REAPPEARANCE OF OPHTHALMIA	DAYS TO MAXIMUM INCREASE IN WEIGHT	DAYS OF SURVIVAL
		*Parenteral**			
1,250 (in 5 days)	Water	Intraperitoneal	30 (25 to 31)	22 (16 to 34)	39† (28 to 63)
1,250 (in 5 days)	Water	Subcutaneous	25 (21 to 30)	21 (13 to 31)	37† (28 to 54)
1,250 (in 5 days)	Oil	Intraperitoneal	36‡ (34 to 38)	38 (25 to 51)	39 (20 to 58)
250	Water	Intraperitoneal	16§	11 (9 to 13)	16 (5 to 26)
		Oral			
1,250 (in 5 days)	Water	Stomach tube	71‖ (61 to 81)	50 (36 to 58)	90 (54 to 110)
50 (in 5 days)	Oil	Dropper	26 (21 to 29)	21 (11 to 29)	30 (24 to 36)
		Parenteral			
Had been 375 γ of carotene per week	Water	Intraperitoneal	21 (15 to 23)	7 (3 to 9)	28 (20 to 35)
750 γ of carotene per week	Water	Intraperitoneal	20 (15 to 28)	17 (8 to 34)	33 (21 to 57)
		Oral			
75 γ of carotene per week	Water	Stomach tube	29 (24 to 34)	13	45 (26 to 64)

*At least 4 rats were used at each level.
†Some rats were killed when losing weight and showing severe ophthalmia, others died.
‡Two rats died without relapse of symptoms.
§Two rats showed no remission of ophthalmia; another died without relapse of symptoms.
‖One rat died without relapse of symptoms.

(b) *Availability of Carotene Deposits.*—The presence of large deposits of injected carotene raised the question of their availability. Accordingly, rats were given large injections of carotene and were then continued on the basal ration with observations on growth, cure of ophthalmia, reappearance of ophthalmia, and time of death. Details of dosage, mode of administration, and results are given in Table II. The liver and various other tissues were analyzed for carotene.

Animals that were given 5 injections totaling 1,250 μg of carotene grew for a short time, and their ophthalmia disappeared (Table II), but within a month ophthalmia reappeared with loss of weight until death supervened. In

all cases unutilized carotene was found in the livers and at the site of injection (Table III). Animals injected with only 250 μg of carotene were variable in therapeutic response, but deposits of carotene were invariably found at death. No essential difference in result was obtained when carotene was injected in oil solution or in colloidal suspension.

TABLE III

CAROTENE FOUND IN RATS AFTER INJECTION OF 1,250 GAMMA OF CAROTENE OVER A FIVE-DAY PERIOD*

METHOD OF ADMINISTRATION	MEDIA	TIME AFTER LAST INJECTION (DAYS)	TOTAL CAROTENE IN LIVER (μg)	CAROTENE AT SITE OF INJECTION (μg)
Intraperitoneal	Water	2	35 (10 to 72)	147 (104 to 183)
Intraperitoneal	Water	30±†	22 (4 to 92)	65 (8 to 121)
Subcutaneous	Water	2	11 (8 to 15)	138 (127 to 151)
Subcutaneous	Water	30±†	4 (traces to 18)	30 (4 to 182)
Intraperitoneal	Oil	30±†	16 (traces to 42)	267 (138 to 352)

*Each value is an average of 3 or more rats.
†Animals had died of vitamin A deficiency.

To determine whether large deposits of injected carotene were more effective biologically than small deposits, we used animals which had been injected with 375 or 750 μg of carotene weekly for five weeks. Carotene deposits averaged 935 μg in the latter (Table I) as compared with 182 μg in animals injected only over the five-day period (Table III). Nevertheless, the behavior of the two groups of animals was essentially the same when they were continued on the basal low vitamin A diet. The restoration of growth and the cure of ophthalmia were temporary, and death occurred in an average of thirty-three days in both groups. Apparently the accumulated carotene was practically unavailable. No carotene was found in the urine or feces after its injection.

PARENTERAL ADMINISTRATION OF VITAMIN A

In view of the inefficient utilization of injected carotene, parallel experiments were performed with vitamin A.

(a) *Physiologic Requirements.*—Vitamin A was injected as an aqueous colloidal suspension or as a solution of halibut-liver oil in cottonseed oil. Seven hundred and fifty blue units injected weekly or fed by stomach tube restored growth, cured ophthalmia, and produced hepatic stores (Table IV). The results were essentially the same whether the colloidal vitamin was injected subcutaneously or intraperitoneally, or fed by stomach tube. When the vitamin was injected in oil solution, however, somewhat better growth resulted than when the colloidal vitamin was injected.

A general consideration of all the results obtained indicated that the efficiency of injected vitamin A was approximately equal to that of vitamin A given orally, as judged by the criteria of growth, cure of ophthalmia, and hepatic storage. At lower levels of administration, 30 and 75 blue units per week, the

biological response depended to some extent on the vehicle in which the vitamin was administered and also on the method of administration. Aqueous colloidal vitamin A was more effective when injected than when given orally (Table IV); oil solutions, on the contrary, were more effective when given by mouth.

TABLE IV

EFFECT OF VITAMIN A ADMINISTERED IN VARIOUS WAYS

LEVEL OF VITAMIN A GIVEN (B.U./WK.*)	MEDIA	METHOD OF ADMINISTRATION	AVERAGE GAIN IN WEIGHT AT 5 WEEKS (GM.)	AVERAGE HEPATIC STORES (B.U.)
		(a) Parenteral†		
750	Water	Intraperitoneal	59	386
750	Water	Subcutaneous	70	358
750	Oil	Intraperitoneal	115	196‡
75	Water	Intraperitoneal	52	0
30	Water	Intraperitoneal	21 (1 dead)	0
30	Oil	Intraperitoneal	14 (2 dead)	0
		(b) Oral		
750	Water	Stomach tube	67§	412
75	Water	Stomach tube	−12 (2 dead)	0
30	Water	Stomach tube	−10 (2 dead)	0
30	Oil	Dropper	63	0

B.U. = Blue unit.
*The vitamin A was administered three times weekly for a five-week period.
†Each value is an average of 4 or more rats.
‡Some vitamin A and oil from the abdominal cavity were unavoidably included.
§One rat responded poorly throughout the experimental period.

The foregoing results may be rationalized on two assumptions: first, that colloidal vitamin A is relatively unstable to the acids of the digestive tract; and secondly, that the vitamin is more readily mobilized from the injected aqueous suspension than from an injected oil solution. The colloidal vitamin A was presumably present as the alcohol as a result of previously saponifying the halibut-liver oil, which contains the vitamin in the form of esters (Hickman[32, 33]). This fact may be noteworthy because vitamin A alcohol and ester are reported to have unequal biological activities when given by mouth (Moll and Reid[34]). It is of interest that Gordon and Titherington[6] observed that vitamin A was somewhat more effective on parenteral administration than when given orally, whereas Greaves and Schmidt[10] reached the opposite conclusion.

In contrast with carotene, most of the injected vitamin A accumulated in the liver rather than at the site of injection. For example, when 750 blue units of colloidal vitamin A were injected intraperitoneally and weekly for five weeks, 105 to 140 units remained in the peritoneal cavity, and 386 units appeared in the liver. On subcutaneous injection 20 blue units remained at the site of injection, and 358 units appeared in the liver. Injected oil solutions, however, persisted at the site of injection or in the abdominal cavity. Lipoidal masses containing 1,125 to 1,797 blue units were found adhering to the liver, although the liver tissue contained relatively little (Table IV).

(b) *Availability of Vitamin A Deposits.*—The question remained whether stores of vitamin A produced by injection could be utilized readily, or whether, like injected carotene, they were inert. Deficient rats, therefore, were given large injections of vitamin A and then continued on the basal diet. Notations were made on the reappearance of ophthalmia and the time of survival (Table V).

Two hundred and fifty blue units of colloidal vitamin A administered in one dose were insufficient to produce hepatic stores, although ophthalmia was cured and growth was promoted for a short time; following this, ophthalmia reappeared, growth failed, and death resulted. The response was essentially the same whether the colloid was given subcutaneously, intraperitoneally, or orally. However, a better response was obtained with injections in oil (Table V). A total of 1,250 blue units of colloidal vitamin produced the following hepatic stores over a five-day period: 142 blue units after intraperitoneal injection, 121 units after subcutaneous injection, and 149 units when fed by stomach tube.

TABLE V

AVAILABILITY OF VITAMIN A STORES OF RATS*

TOTAL VITAMIN A GIVEN (B.U.)	MEDIA	METHOD OF ADMINISTRATION	AVERAGE DAYS TO REAPPEARANCE OF OPHTHALMIA	SURVIVAL PERIOD (DAYS)
		(a) Parenteral		
1,250 (in 5 days)	Water	Intraperitoneal	79† (76 to 81)	88 (63 to 108)
1,250 (in 5 days)	Water	Subcutaneous	64 (44 to 93)	87 (71 to 113)
250 (1 injection)	Water	Intraperitoneal	27 (13 to 46)	38 (13 to 75)
250 (1 injection)	Water	Subcutaneous	37‡ (32 to 40)	39 (15 to 69)
250 (1 injection)	Oil	Intraperitoneal	49† (31 to 63)	62 (39 to 88)
		(b) Oral		
1,250 (5 doses)	Water	Stomach tube	53† (47 to 60)	71 (58 to 79)
250 (1 dose)	Water	Stomach tube	24§ (13 to 40)	36 (18 to 62)

*Each value is an average of 4 or more rats.
†One animal died without relapse of symptoms.
‡Two animals died without relapse of symptoms.
§One animal showed no remission of ophthalmia.

Despite the similarity in the stores produced, the vitamin appeared to be more effective physiologically when injected than when given orally; injected animals lived for eighty-eight days as compared with seventy-one days for those given the vitamin orally (Table V). Ophthalmia was deferred for seventy-two days in the injected animals as compared with fifty-three for the latter. At death no stores of vitamin A were found either in the liver or at the site of injection. Vitamin A administered parenterally, therefore, was apparently utilized at least as efficiently as when given orally.

SUMMARY

Rats deficient in vitamin A were injected intraperitoneally or subcutaneously with carotene in aqueous colloidal suspensions or in oil solution. Growth was restored and symptoms of ophthalmia were cured, but the amount needed was ten to one hundred times as great, and the effect was less prolonged than when carotene was given orally. Parenteral carotene failed to yield stores of hepatic vitamin A even when 1.5 mg. were injected weekly for five weeks. Particles of injected carotene were found at the sites of injection, and also in the liver, omentum, and lymph nodes in the peritoneal cavity. Carotene in these areas was only partially available to the animal, since deficiency symptoms and death occurred while considerable amounts of carotene were still in evidence.

Much of the injected carotene could not be traced; it was not converted into vitamin A nor was it found in the excreta.

Vitamin A, in contrast to carotene, was utilized about as effectively when injected as when given orally. Aqueous colloidal preparations were utilized best when injected; oil solutions were utilized best when given orally.

We wish to express our appreciation to Lever Brothers Company whose financial support made this work possible.

REFERENCES

1. Wollman, E., and Vogliano, M.: Action de la vitamin de croissance A administrée par voie parenterale, Compt. rend. Soc. de biol. **88**: 336, 1923.
2. Koehne, M., and Mendel, L. B.: The Utilization of Fatty Oils Given Parenterally, J. Nutrition **1**: 399, 1929.
3. Wolff, L. K., Overhoff, J., and van Eckelen, M.: Ueber Carotin und vitamin A, Deutsche med. Wchnschr. **56**: 1428, 1930.
4. von Euler, H., and Rydbom, M.: Zur kenntnis der vitaminwirkungen von carotin, Arkiv. Kemi, Mineralogie och Geologi **10B**: 1, 1930.
5. Rydbom, M.: Versuche über die Wachstumswirkung von Carotinoiden, Biochem. Ztschr. **227**: 482, 1930.
6. Gordon, B., and Titherington, R. J.: The Effect of Cod Liver Oil Concentrate Injections, Am. Rev. Tubere. **27**: 368, 1933.
7. Chu, F., and Coady, H.: Parenteral Use of Carotene in the Treatment of Vitamin A Deficiency in Albino Rats, J. Pediat. **5**: 75, 1934.
8. Lasch, F.: Vitamin A-stoffwechsel und leber bei experimenteller phosphorvergiftung, Klin. Wchnschr. **14**: 1070, 1935.
9. Greaves, J. D., and Schmidt, C. L. A.: On the Absorption and Utilization of Carotene and Vitamin A in Choledochocolonostomized Vitamin A Deficient Rats, Am. J. Physiol. **111**: 492, 1935.
10. Greaves, J. D., and Schmidt, C. L. A.: The Utilization of Carotene by Jaundiced and Phosphorus Treated Vitamin A Deficient Rats, Am. J. Physiol. **111**: 502, 1935.
11. Guilbert, H. R., and Hart, G. H.: Minimum Vitamin A Requirements With Particular Reference to Cattle, J. Nutrition **10**: 409, 1935.
12. Wilson, H. E. C., Ahmad, B., and Majumdar, B. N.: Further Observations on the Metabolism of Carotene, Indian J. M. Research **24**: 399, 1936.
13. Phillips, P. H., and Bohstedt, G.: Studies on the Effects of a Bovine Blindness-Producing Ration Upon Rabbits, J. Nutrition **15**: 309, 1938.
14. Blegvad, O.: The Parenteral Vitamin Treatment of Xerophthalmia, Ugesk. f. laeger. **85**: 942, 1923. From Brit. M. J. **1**: 122, 1924.
15. Edmund, C.: Über hemeralopia idiopatica mit besonderem hinblick auf untersuchung und behandlung, Acta. ophth. **2**: 225, 1924. Quoted in Acta. med. Scandinav. **89**: 69, 1936.
16. Bloch, C. E.: Further Clinical Investigations Into the Diseases Arising in Consequence of a Deficiency in the Fat-Soluble A Factor, Am. J. Dis. Child. **28**: 659, 1924.
17. Bloch, C. E.: Light and the A-Body, Am. J. Dis. Child. **31**: 315, 1926.
18. Buschke, W.: Merkwürdiger Krankheitsverlauf bei einem mit Carotin behandelten Fall von Ikterus mit Nachtblindheit, Schweiz. med. Wchnschr. **67**: 777, 1937.
19. Josephson, E. M., and Freiberger, M.: Carotene Therapy of Retinitis Pigmentosa, Nature, London **139**: 155, 1937.
20. Funk, C.: The Vitamines, p. 227, 1922.
21. Rea, J. L., and Drummond, J. C.: On the Formation of Vitamin A From Carotene in the Animal Organism, Ztschr. f. Vitaminforsch. **1**: 177, 1932.
22. Drummond, J. C., and MacWalter, R. J.: The Biological Relation Between Carotene and Vitamin A, Biochem. J. **27**: 1342, 1933.
23. Drummond, J. C., and MacWalter, R. J.: The Fate of Carotene Injected Into the Circulation of the Rat, J. Physiol. **83**: 236, 1935.
24. Drummond, J. C., Gilding, H. P., and MacWalter, R. J.: Fate of Carotene Introduced Into the Circulation, J. Physiol. **82**: 75, 1934.
25. Ahmad, B., Grewal, K. S., and Malik, K. S.: Further Observations on the Metabolism of Carotene, Indian M. Gaz. **69**: 320, 1934. From Biol. Abst. **11**: 610, 1937.
26. Edmund, C., and Clemmesen, S.: On Parenteral A Vitamin Treatment of Dysaptatio (Nyctalo-hemeralopia) in Some Pregnant Women, Acta. med. Scandinav. **89**: 69, 1936.
27. Finkelstone, B. B., Howard, J. H., and Paris, M.: Intramuscular Injection of Vitamins A and D Concentrates During Pregnancy, Am. J. Obst. & Gynec. **32**: 113, 1936.
28. Decourt, M. J.: Osteomalacie grave non influencée par l'ingestion de vitamin A, mais considerablement ameliorée par l'administration de vitamines A et D en injections intramusculaires, Bull. et mém. Soc. méd. d. hôp. de Paris **53**: 248, 1937.
29. Hawk, P. B., and Oser, B. L.: A Modification of the Osborne-Mendel Salt Mixture, Science **74**: 369, 1931.

30. Carr, F. H., and Price, E. A.: Color Reactions Attributed to Vitamin A, Biochem. J. **20**: 479, 1926.
31. van den Bergh, H., Muller, P., and Broekmeyer, J.: Das Lipochrompigment in Blutserum und Organen. Xanthosis und Hyperlipochromänie, Biochem. Ztschr. **108**: 279, 1920.
32. Hickman, K. C. D.: The Identification of Vitamins by Molecular Distillation, Nature **138**: 881, 1936.
33. Hickman, K. C. D.: Molecular Distillation. State of the Vitamins in Certain Fish Liver Oils, J. Ind. Eng. Chem. **29**: 1107, 1937.
34. Moll, T., and Reid, A.: Über die verschiedene Wirksamkeit von Vitamin A-Konzentraten aus Fischleberölen, Ztschr. f. physiol. Chem. **260**: 9, 1939.

THE INFLUENCE OF A PAROTID EXTRACT ON THE BLOOD SUGAR AND STRUCTURE OF THE PANCREAS OF THE RAT*

WILLIAM B. BIRNKRANT, M.S., M.D., NEW YORK, N. Y.
ASSISTED BY REBECCA SHAPIRO, M.S.

THE role of the parotid gland in carbohydrate metabolism has been the subject of controversy. Blood sugar-raising substances have been obtained from the parotid gland by Hiki and co-workers.[1] Takaes,[2] however, reported hypoglycemias following the injection of ether and alcohol extracts of this gland. Birnkrant[3] demonstrated marked hypoglycemias in rats following total bilateral parotidectomies. Cahane and Cahane[4] described hypertrophy and hyperplasia of the islands of Langerhans following bilateral parotidectomies on two dogs. Aunap,[5] Dobrzaniecki, and Michalowski,[6] reported diminution of hyperglycemias following parotidectomies in depancreatized dogs. Zibordi[7] and Scollo[8] have also demonstrated a relationship between the pancreas and the parotid gland in their experimental animal work.

There also has been some clinical evidence to support a parotid-pancreas relationship. Of Flaum's[9] 27 patients with bilateral hypertrophy of the parotid gland, 16 had frank diabetes and 11 had incipient diabetes. He considers every patient presenting noninflammatory enlargements of the parotid gland a diabetic unless proved otherwise. Kenawy[10] found approximately 2 per cent of the poorer agriculture classes in Egypt suffering from a chronic, painless, noninfectious, noninflammatory enlargement of the parotid gland. Ten per cent of the cases he studied were diabetics. All presented bilateral swellings.

Both Ferretti[11] and Dobreff[12] stated that hypertrophy of the parotid glands is a result of a compensatory mechanism, i.e., the parotid glands take on the function of the pancreas in some instances of hypoinsulinism. On the other hand, the Cahanes, and Dobrzaniecki and Michalowski interpreted their findings on the basis of an anti-insulin parotid hormone.

Although the work of these authors indicates a carbohydrate-regulating mechanism related to the parotid gland, apparently conflicting reports and opposing interpretations have made difficult a proper evaluation of this mechanism.

The present study was, therefore, undertaken to determine the effects of parotid extracts on tissues and on blood sugar levels. Blood-sugar raising sub-

*From the Laboratories of the Delamar Institute of Public Health, Columbia University.
Received for publication, December 4, 1940.

stances have been prepared from the urine of diabetic patients by Werch and Altshuler,[13] and by Harrow and associates.[14] In order to determine whether a relationship exists between these blood-sugar raising substances isolated from the urine of diabetic patients and the parotid extracts used by the earlier workers, a parotid extract was prepared by modifying the method of Harrow and others in order to make it suitable for tissue work.

EXPERIMENTAL TECHNIQUES

(A) *Preparation of Extract.*—To 1,250 Gm. of fresh ground beeves' parotid gland from which the fat and fascia had been removed, 2,250 c.c. of distilled water were added. The mixture was digested for twelve hours at 10° C. in three-eighths its volume of 0.2 N sodium hydroxide, then filtered and centrifuged. The supernatant fluid was acidified to pH 5 with glacial acetic acid. Sixty cubic centimeters of 95 per cent alcohol, containing 30 Gm. of benzoic acid, were added for each liter of solution. The precipitate was sucked dry on a Buchner funnel, washed with alcohol and ether, dried in vacuo and weighed (precipitate I, weight 132 Gm.).

The crude extract was triturated with 12 c.c. of distilled water per gram and dialyzed at 4° to 10° C. for three days. The contents of the dialyzing membrane were evaporated in vacuo to a small volume and centrifuged. The dried sediment (precipitate II) weighed 104 Gm.

To the supernatant fluid from this centrifuged mixture, absolute alcohol was added to make a final 95 per cent solution. This solution was kept at 4° to 10° C. for thirty-six hours and then centrifuged. The sediment was washed with alcohol and ether and dried (precipitate III, weight 320 mg.). Precipitate III was composed of thin, highly refractile, monoclinic, tetragonal crystals, soluble in water and dilute alcohol, insoluble in absolute alcohol and ether, and partially soluble in acetone. It turned brown and gummy on standing.

(B) *Blood Sugar Determinations.*—Precipitates I, II, and III were injected intraperitoneally into male Long-Evans hooded rats, eight to twelve weeks old and weighing from 207 to 305 Gm. The rats were kept on a well-balanced constant diet. Blood samples were secured from the palmar veins. The modified microcolorimetric method of Benedict[15] was used for the sugar determinations. This method determines nonfermentable substances equivalent in reducing power to only 4 to 8 Gm. of glucose per 100 c.c. of blood.

For the glucose tolerance tests 1 Gm. of glucose per kilogram of rat weight was fed orally in a 50 per cent solution to the fasting rat. The rats were previously conditioned to medicine dropper feedings.

Six or more blood sugars were determined on each rat prior to the experimental procedures in order to establish normal means.

EXPERIMENTAL PROCEDURES AND RESULTS

(A) *Parotid Extracts.*—1. Precipitate I. Seventy-five milligrams of precipitate I were injected intraperitoneally into four fasting rats. Seven days later two of these rats were again injected with the same amount of extract, and fourteen days later one was reinjected. Fasting blood sugar levels were determined, and blood samples were secured in one hour, four hours, and twenty-four hours after injection.

In one hour the blood sugars showed an average increase of 87 per cent over the fasting blood sugars, ranging from 163 mg. per cent to 216 mg. per cent (Table I), representing a markedly significant rise (the sigma of the difference equals 12). Four hours after injection the blood sugars were still significantly higher than those of the fasting levels. In twenty-four hours all the blood sugars had returned to the preinjection range.

TABLE I

EFFECTS OF PRECIPITATE I ON BLOOD SUGARS

RAT NO.	MEANS OF SIX BLOOD SUGARS PRIOR TO INJECTION	FASTING BLOOD SUGAR PRIOR TO INJECTION	PRECIPITATE I INJECTED (MG.)	BLOOD SUGARS AFTER INJECTION (MG. %)		
				1 HR.	4 HR.	24 HR.
14	82± 4	90	75	171	146	121
36	91± 6	85	75	174	116	88
35	91± 3	103	75	163	157	109
Test repeated 7 days later	91± 3	104	75	166	130	97
30	106± 4	92	75	216	149	95
Test 7 days later	106± 4	108	75	188	139	94
15 days later	106± 4	97	75	171	149	94
Mean	95± 2	97± 3		178± 7	141± 6	100± 4
Mean increase				83± 7	46± 6	
Mean per cent increase				87	45	

TABLE II

EFFECTS OF PRECIPITATE II ON BLOOD SUGARS

RAT NO.	MEANS OF SIX BLOOD SUGARS PRIOR TO INJECTION	AMOUNT INJECTED (MG.)	BLOOD SUGARS AFTER INJECTION (MG. %)		
			1 HR.	4 HR.	12 HR.
3	94± 5	50	139	148	123
14	82± 5	50	155	130	122
15	100± 4	50	164	117	126
29	93± 6	50	130	90	117
30	106± 4	50	128	103	109
51	93± 4	50	146	115	128
55	88± 5	50	166	106	123
60	97± 5	50	146	119	110
62	90± 5	50	160	108	110
Mean	93± 1		150± 7	117± 6	118± 3
Mean increase			57± 7	24± 6	25± 3
Mean per cent increase			61	26	27

2. Precipitate II. Fifty milligrams of precipitate II were injected intraperitoneally into each of ten rats daily for eight days. Samples of blood were secured on the eighth day immediately prior to the injection. Thereafter, one-hour, four-hour, and twelve-hour fasting samples were drawn from each rat. In one hour the blood sugars showed a 61 per cent average increase. The four-hour sample was 26 per cent above the mean blood sugars prior to the series of injections. Twelve hours after injection the same average increase was recorded (Table II). These average increases in blood sugars were statistically significant.

Eighteen glucose tolerance tests were done on 11 rats after they had received from one to twelve consecutive daily injections of 100 mg. of precipitate II (Table III). Fasting blood samples were secured immediately before injection. Fourteen rats were injected with 100 mg. of precipitate II just prior to the glucose tolerance test. Four rats were not injected immediately prior to the test, but received their last injection twenty-four hours previously. Two tests were done after one injection, two after three daily injections, eight after eight daily injections, four after nine daily injections, and two after twelve daily injections.

TABLE III

GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE II

RAT NO.	NO. OF CONSECUTIVE DAILY INJECTIONS	MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS	FASTING BLOOD SUGARS	PRECIPITATE II PRIOR TO TEST (MG.)	BLOOD SUGARS FOLLOWING INJECTION (MG. %)				
					½ HR.	1 HR.	1½ HR	2 HR.	3 HR.
49	1	97± 4	97	100	160	216	196	202	128
2	1	93± 2	94	100	189	169	158	139	128
15	3	100± 4	106	100	153	178	162	146	108
14	3	82± 5	117	100	171	162	160	128	115
14	8	82± 5	86	100	585	207	---	146	101
15	8	100± 4	121	0	191	231	157	167	137
55	8	88± 5	128	100	502	166	133	---	135
46	8	93± 7	140	100	203	270	265	266	272
20	8	108± 5	131	100	207	162	148	144	137
3	8	94± 5	119	100	304	---	225	265	153
51	8	93± 4	131	100	191	216	146	146	131
61	8	101± 7	148	100	198	171	151	149	139
3	9	94± 5	128	0	183	---	480	136	131
15	9	100± 4	130	100	---	250	---	169	104
51	9	93± 4	126	100	165	---	144	---	166
62	9	90± 5	146	100	178	157	148	157	138
14	12	82± 5	132	0	148	---	142	133	185
55	12	88± 5	119	0	241	155	137	126	146

In order to control the results eight glucose tolerance tests were done on rats not injected with the parotid extracts. Four of these tests were done after eight daily injections of physiologic saline, and two after eight daily beef extract broth injections. Two were not injected (Table IV).

The results of the control rats closely simulated glucose tolerance curves in normal human beings. Practically all the blood sugar curves following the use of precipitate II presented a picture similar to that in diabetic patients (Table III). The one-half-hour samples of the latter ranged from 148 mg. per cent to 585 mg. per cent, whereas the one-half-hour samples of the control animals ranged from 117 mg. per cent to 153 mg. per cent. The one-hour test samples of the rats injected with the parotid precipitate ranged from 155 mg. per cent to 270 mg. per cent, whereas that of the controls ranged from 101 mg. per cent to 128 mg. per cent. In the one-and-one-half-hour samples blood sugars ranging from 133 mg. per cent to 480 mg. per cent were obtained in the injected animals, in comparison to blood sugars ranging from 90 mg. per cent to 131 mg. per cent in the control animals. All the two-hour blood sugars of the control animals were normal, whereas the two-hour samples of the injected animals ranged

from 126 mg. per cent to 266 mg. per cent. The three-hour samples from the injected animals ranged from 101 mg. per cent to 272 mg. per cent (Tables III and IV).

TABLE IV

GLUCOSE TOLERANCE TESTS OF CONTROL RATS

RAT NO.	MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS	FASTING BLOOD SUGARS	SUBSTANCE USED	AMOUNT INJECTED (C.C.)	BLOOD SUGARS FOLLOWING INJECTION (MG. %)				
					½ HR.	1 HR.	1½ HR.	2 HR.	3 HR.
60	97± 5	94	Physiol. saline	3	139	126	104	99	83
61	101± 7	102	Physiol. saline	3	130	124	121	103	103
62	90± 5	115	Physiol. saline	3	153	126	131	110	103
63	103± 4	99	Physiol. saline	5	145	128	115	126	102
33	99± 5	117	Beef extract broth	5	139	122	113	94	104
4	91± 5	79	Beef extract broth	5	131	118	115	119	88
64	105± 5	79	0	0	117	103	124	99	94
20	108± 5	101	0	0	121	101	90	97	79
Mean	99± 2	103± 5			134± 4	119± 4	114± 5	106± 4	95± 4

TABLE V

GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE II EXCLUSIVE OF TESTS SHOWING EXTREME OR SECONDARY PEAKS

RAT NO.	NO. OF CONSECUTIVE DAILY INJECTIONS	MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS	FASTING BLOOD SUGARS	PRECIPITATE II PRIOR TO TEST (MG.)	BLOOD SUGARS FOLLOWING INJECTION (MG. %)				
					½ HR.	1 HR.	1½ HR.	2 HR.	3 HR.
49	1	97± 4	97	100	160	216	196	202	128
2	1	93± 2	94	100	189	169	158	139	128
14	3	82± 5	117	100	171	162	160	129	115
15	3	100± 4	106	100	153	178	162	146	108
20	8	108± 5	131	100	207	162	148	144	137
46	8	93± 7	140	100	203	270	265	266	272
15	8	100± 4	121	0	191	237	157	167	137
61	8	101± 7	148	100	198	171	151	149	139
51	8	93± 4	132	100	191	216	146	146	131
15	9	108± 4	130	100	---	250	---	169	104
Mean	–	97± 1	122± 6		185 ±6	203 ±13	171± 4	166 ±12	140 ±15
Mean increase over control rats					51 ±8	84 ±14	57± 6	60 ±13	45 ±18
Per cent increase over control rats			18		38	71	50	57	47

Three distinct types of curves were obtained. The most frequently encountered (Table V) reached its peak within one hour, with an average 71 per cent higher than that of the controls. The blood sugars of the control animals uniformly reached a peak in one-half hour. There was a 47 per cent increase in the three-hour samples over those of the controls. The average of the three-

hour blood sugars of the test animals was higher than that of their fasting blood sugars; whereas the average of the three-hour samples of the control rats was lower than the average of their fasting samples (Tables IV and V). All the average increases in the blood sugars of the test animals were statistically significant.

The second type of curve was encountered in Rats 3, 55, and 14 after eight daily injections, and in Rat 3 after nine daily injections. Extreme blood sugar rises from 304 to 585 mg. per cent were obtained in the one-half hour to the one and one-half hour samples (Table III). Sharp drops in the following samples followed these extreme peaks. Secondary peaks in the two- or three-hour blood samples were encountered in the tolerance test of six rats.

Although the majority of the tolerance curves paralleled the first type, the rats did not react constantly with any one specific type.

3. Precipitate III. Five milligrams of precipitate III were injected into seven rats. The mean of the blood sugars one hour after injection was 57 per cent higher than that of the mean of 42 blood sugars prior to the injections, representing a highly significant increase. The mean of the three-hour blood sugars was 25 per cent higher than that of the control sugars (Table VI).

TABLE VI

EFFECTS OF PRECIPITATE III ON BLOOD SUGARS

RAT NO.	MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS	AMOUNT INJECTED (MG.)	BLOOD SUGARS AFTER INJECTIONS (MG. %)	
			1 HR.	3 HR.
29	93± 6	5	176	115
52	96± 2	5	157	113
51	93± 4	5	149	124
55	88± 4	5	148	114
54	94± 6	5	138	110
15	100± 4	5	146	124
21	104± 5	5	142	134
Mean	96± 2		151± 5	120± 3
Mean increase			55± 5	24± 4
Per cent increase over controls			57	25

TABLE VII

GLUCOSE TOLERANCE TESTS OF RATS INJECTED WITH PRECIPITATE III

RAT NO.	NO. OF CONSECUTIVE DAILY INJECTIONS	MEAN OF SIX BLOOD SUGARS PRIOR TO INJECTIONS	FASTING BLOOD SUGARS	PRECIPITATE III PRIOR TO TEST (MG.)	BLOOD SUGARS FOLLOWING INJECTION (MG. %)				
					½ HR.	1 HR.	1½ HR.	2 HR.	3 HR.
51	1	93± 4	94	3	202	128	149	151	171
61	1	101± 7	74	3	128	128	151	185	106
62	1	90± 5	110	3	148	160	146	131	108

Three glucose tolerance tests (Table VII) after injection of 3 mg. of precipitate III were similar to those obtained after the injection of 100 mg. of precipitate II.

(B) *Tissues.*—Two rats were autopsied after having received eight daily injections of beef extract broth. Seven rats were autopsied after nine to twelve daily injections of precipitate II, and in addition one injection of precipitate III.

The pancreas of the rats injected with beef extract broth presented no changes in the parenchyma of the islands of Langerhans.

The parenchyma of the pancreas of those rats which had received the injections of the several parotid extracts was normal. The majority of the islands of Langerhans were normal in size, shape, and arrangement of the island cells. In all the specimens, however, there were islands exhibiting marked granular degeneration, hydropic degeneration, disorganization of the normal island pattern, and varying degrees of infiltration with small round cells and fibroblasts. In several islands there was almost complete replacement of the island cells with young fibroblasts. Sections of the parotids, livers, spleens, kidneys, and thyroid glands of the extract injected animals revealed no pathologic changes.

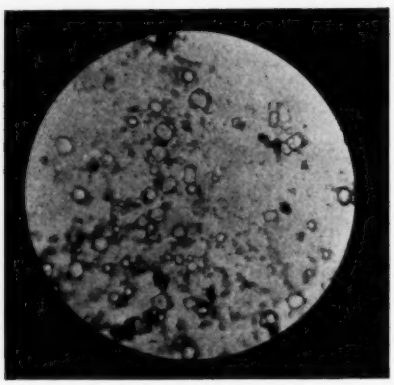


Fig. 1.

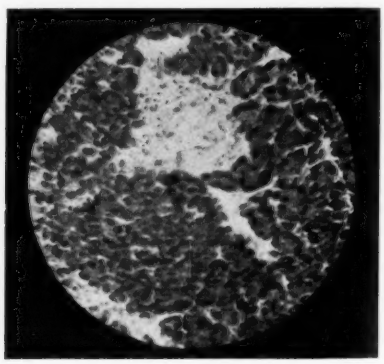


Fig. 2.

Fig. 1.—Crystals of precipitate III (×320).
Fig. 2.—Replacement of island cells with fibroblasts (×320).

DISCUSSION

The constant and prolonged hyperglycemias following intraperitoneal injections of the parotid extracts indicate the existence of an insulin antagonist secreted by the parotid gland. The markedly significant 84 per cent increase in the one-hour samples following the injection of precipitate I into seven fasting rats may possibly be explained by assuming that glycogenesis was being inhibited, whereas glycogenolysis was proceeding at a normal rate, thus resulting in an abnormal accumulation of endogenous glucose in the blood.

Injections of an average of only 5 mg. of precipitate III into seven rats produced an average increase of 57 per cent in the one-hour samples. Similar increases were obtained following the use of precipitate II in nine rats. Injections of 5 mg. of the refined crystalline precipitate III produced hyperglycemias comparable to those following 50 mg. of precipitates I and II (Tables I, II, and VI). We cannot, however, state that precipitate III was ten times as potent as precipitates I and II, since the end point of the inhibiting effects of the extract has not yet been determined. A chemical analysis of the crystals constituting precipitate III is still in progress.

The blood sugars of rats that received the parotid extract for the first time returned to the preinjection level within twenty-four hours, whereas the fasting blood sugars of those rats that received repeated daily injections were 31 per cent greater than their preinjection average.

These findings demonstrate that, although the inhibiting effect of the parotid substance disappears within twenty-four hours after one massive dose, repeated injections tend to maintain the fasting blood sugars at higher levels, even after the cessation of the injections due to the degenerative changes in the islands of Langerhans. This effect is exemplified by the glucose tolerance tests of Rats 15, 3, 14, and 55 (Table III), in which occurred peaks of 231, 480, 185, and 241 mg. per cent, twenty-four hours after the last injection of parotid extract. The normal glucose tolerance curves of control Rats 4 and 33, that had been repeatedly injected with beef extract broth, make improbable the possibility that a nonspecific irritating effect of intraperitoneal injections of a tissue extract may have resulted in hyperglycemia. Sections of the pancreas of these rats revealed no abnormal changes. Likewise, the normal glucose tolerance curves of the four rats which received repeated intraperitoneal injections of physiologic saline solution exclude the possibility that the hyperglycemias in the test animals were the result of repeated peritoneal punctures.

It is difficult in the present stage of this study to determine whether the degenerative changes in the islands of Langerhans following injections of the parotid extracts represent a reaction to a specific cell toxin, or are the end results of an accelerated type of atrophy of disuse following prolonged inhibition of all function.

Wereh and Altshuler, and Harrow and co-workers, prepared blood-sugar raising substances from the urine of diabetic patients. The former found greater amounts of this substance in the urine of persons with diabetes than in the urine of normal individuals, and even greater amounts in urine in uncontrolled diabetes than in that of controlled diabetes. In the present study the method used by Harrow and associates to extract blood-sugar raising substances from the urine was applied in a modified form to the parotid gland. A potent blood-sugar raising extract was secured.

Himsworth[16] in 1936 suspected the presence of an insulin antagonist. He suggested that diabetes mellitus in the insulin-sensitive person was due to a lack of insulin, whereas diabetes in the insulin-insensitive person was due to an insulin antagonist. It is possible that his two types of patients with diabetes represent two different stages of a condition with the same etiologic mechanism. An island of Langerhans antagonist may cause primary effects through its specific insulin antagonism and secondary effects following permanent damage to the islands of Langerhans. This damage may be due to either an atrophy of disuse, or a specific cell toxin.

SUMMARY

1. A potent blood-sugar raising extract was prepared from the parotid gland.

2. Degenerative changes in the islands of Langerhans followed repeated injections of this extract in experimental animals.

REFERENCES

1. Hiki, Y., Akazaki, K., Bann, T., Miyazaki, Y., and Takizawa, N.: Experimentelle Untersuchungen der Mundspeicheldrussen, Tr. Jap. Path. Soc. **22**: 273, 1932.
2. Takacs, L.: Internal Secretion of the Parotid Gland, Orvosi hetil. **77**: 659, 1933.
3. Birnkrant, W. B.: The Influence of the Parotid Gland on Blood Sugar, J. Lab. Clin. Med. **26**: 1009, 1941.
4. Cahane, M., and Cahane, F.: La fonction endocrine des glandes salivaires, Rev. franç. d'endocrinol. **8**: 398, 1933.
5. Aunap, E.: Ueber die Beziehungen zwischen Kern, Ergasto-Plasma und Mitrochondriender Parotiszellen der Ratte, Ztschr. f. mikr.-anat. Forsch. **24**: 412, 1931.
6. Dobrzaniecki, W. D., and Michalowski, E.: Influence de la suppression de l'ecretion de la parotide sur la glycogenesis, E. Lyon. Chir. **28**: 571, 1931.
7. Zibordi, F.: Rapporte tra parotide e tasso glicemico, Med. ital. **12**: 78, 1931.
8. Scollo, G.: L'influenza della parotide sul ricambio degli idrate di carbonis e sue diabete pancreatico sperimentale, Policlinico (sez chir.) **40**: 774, 1933.
9. Flaum, E.: Parotishypertrophie ein Symptom des Diabetes Mellitus, Klin. Wchnschr. **11**: 1704, 1932.
10. Kenawy, M. R.: Endemic Enlargement of the Parotid Gland in Egypt, Tr. Roy. Soc. Trop. Med. & Hyg. **31**: 339, 1937.
11. Ferretti, G.: Su due casi di ipertrofia, delle parotidi in diabetici, Gior. di clin. med. **17**: 1149, 1936.
12. Dobreff, M.: Compensatory Hypertrophy of the Parotid Gland in Presence of Hypofunction of Pancreatic Islands, Deutsche med. Wchnschr. **62**: 67, 1936.
13. Werch, S. C., and Altshuler, S. S.: A Study of the Blood Sugar Raising Substance in the Urine of Diabetics and Non Diabetics, Am. J. Physiol. **118**: 659, 1937.
14. Harrow, B., Chamelin, J. M., Mazur, A., and Leserk, C.: Concentration of a Hyperglycemic Factor From Urine, Proc. Soc. Exper. Biol. & Med. **34**: 688, 1936.
15. Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and Saccharoides (Non-fermentable Copper Reducing Substances), J. Biol. Chem. **92**: 141, 1931.
16. Himsworth, H. P.: Diabetes Mellitus, Its Differentiation Into Insulin Sensitive and Insulin Insensitive Types, Lancet **1**: 127, 1936.

14 Fifth Avenue

THE ORAL ASCORBIC ACID TOLERANCE TEST AND ITS APPLICATION TO SENILE AND SCHIZOPHRENIC PATIENTS*

Elmer Stotz, Ph.D., Burton M. Shinners, M.D., and Rupert A. Chittick, M.D., Boston, Mass.

IT IS well known that there is a wide difference in the amount of ascorbic acid intake necessary to prevent the outward manifestation of scurvy and the amount desirable for optimal health.[1] As evidence accumulates concerning the true physiologic functions of vitamin C, such as the work of Sigal and King[2] on vitamin C levels and resistance to bacterial toxins, and of Ecker and coworkers[3] on the relation between ascorbic acid and guinea pig complement, there will be an increasing need of a simple clinical procedure for determining the status of vitamin C nutrition in patients. The many techniques already available are well reviewed by authorities in this field in *The Vitamins*, edited in 1939 by the American Medical Association. Determination of the "resting level" of excretion[4-6] and of "saturation deficits"[7, 8] involve so many difficulties

*From the Biochemical Laboratory of the McLean Hospital, Waverley, Mass., and the Department of Biological Chemistry, Harvard Medical School, Boston.

Received for publication, December 9, 1940.

of performance and interpretation, not the least of which is the long time necessary for the test, that these techniques have not found general adoption in the clinic.

The significance of the plasma ascorbic acid content in single fasting specimens has been considered by many[9-12] to be of very limited value as an index of vitamin C nutrition, a belief that is further confirmed by our work. The lack of agreement[13, 14] concerning the clinical significance of such fasting values is itself evidence of the inadequacy of this otherwise simple test. Butler and Cushman[15] have found that the white blood cells and platelets may retain most of their ascorbic acid content even when the plasma ascorbic acid has been reduced to zero, and are themselves reduced to low ascorbic acid values only in acute scurvy. These authors believe that ascorbic acid analyses of these cells "provide indices of vitamin C deficiency which extend beyond the limit of the index furnished by fasting plasma concentrations." Their data indicate, however, that the vitamin C content of these cells is nearly maximal at low plasma concentrations, so that while analysis of these cells may indicate the severity of an existing scurvy, it would not be as sensitive an index of less severe vitamin C unsaturation.

Since the basic interest in "saturation tests" is the existing vitamin C content of the tissues, and since this substance appears to be in the free state both in the tissues and plasma, a reliable index should be furnished by the rate of penetration of the vitamin from the plasma into the tissues. Thus upon feeding the vitamin, if the tissue ascorbic acid content were low, the rapid flow of ascorbic acid from the plasma to such tissues should result in only a small rise of the plasma ascorbic acid; if the tissues were "saturated," then the plasma content should increase markedly. These suppositions were found to be true. Such a tolerance curve with vitamin C administered both orally and intravenously has been used in conjunction with other "saturation tests" by Portnoy and Wilkinson[11] in a study of normal patients and those with ulcer. The test was not studied in detail by these authors, however, and no attempt was made to relate the various degrees of saturation with the type of tolerance curve obtained. Intravenous "test dose" methods with measurement of the urinary excretion have been suggested[16-19] to eliminate possible errors due to differences in intestinal absorption rates. Intravenous administration must in our opinion, and in agreement with that of van Eekelen and Heinemann,[20] involve a sudden flooding of the kidney and loss of a large amount of ascorbic acid, so that the blood values and urinary excretions observed must be greatly influenced by the speed of injection and secretory ability of the kidney. In the "oral" test to be described, the dosage was so chosen that rarely more than 10 to 15 per cent of the total dose was excreted during the five-hour test. Hence the principal factor in determining differences in the curves appeared to be the fundamental one, namely, the rate of penetration of the vitamin from the plasma into the tissues. The shape of an individual curve is, of course, determined in large part by the rate of intestinal absorption, as is the case with a sugar tolerance curve, but in our series we have not encountered any cases in which this factor appeared to be the main determinant of *differences* in the shape of a group of oral tolerance curves. Proof of this fact lies chiefly in the satisfactory explanation of differ-

ences on the basis of vitamin C nutrition alone, and on those changes produced by experimental feeding and deprivation of the vitamin in a single individual, where the factor of gastrointestinal absorption must be considered to remain constant. If cases of exceptionally slow absorption were encountered employing this test, the curve would at least suggest deficiency and lead to vitamin C therapy, and if this failed to produce a "saturated" type of curve, other methods of administration for therapy would be indicated.

This paper includes a study of (a) the use of the oral ascorbic acid tolerance test for a rapid clinical diagnosis of the degree of subclinical vitamin C deficiency; (b) the changes produced in the curve upon experimentally saturating and depleting the same individual of vitamin C; (c) the analysis of sufficient tolerance curves to distinguish at least four "types" characteristic of various degrees of vitamin C nutrition; and (d) the application of the method to a study of schizophrenic and senile patients.

METHOD

The test is best performed in the morning. A sample of blood (approximately 7 c.c.) from a fasting patient is taken with a dry syringe and ejected into a dry pyrex tube containing approximately 20 mg. of potassium oxalate as an anticoagulant. Mixing is accomplished by gentle inversions rather than by vigorous shaking to avoid hemolysis. A dose of 6.0 mg. of ascorbic acid per kilogram of body weight is dissolved (immediately before feeding) in one-half glass water and fed, the time in minutes being noted. Since no food should be given for the following five hours, we have allowed, after the vitamin C, a breakfast consisting of a cup of coffee and plain toast. This did not materially affect the resultant curve, but made the test more tolerable to the patient. Although in our experimental work we have taken five more specimens of blood to determine the details of the curve, sufficient information for clinical tests may be obtained by taking blood specimens at only the two-and-one-half- and five-hour points. The blood samples should be chilled immediately after drawing until they are analyzed (the same day). The chemical determinations of plasma ascorbic acid were done by a method developed in this laboratory[21] which proved to be a rapid clinical method for serial analysis. It is a modification of the Mindlin and Butler procedure,[22] with extraction of the unchanged 2,6 dichlorophenolindophenol into xylene, which insures its stability.

The values obtained in milligrams per cent are plotted against time in minutes.

INTERPRETATION OF CURVES

For the sake of clarity of presentation and economy of space it would seem better to present first a chart summarizing the types of curves which have been distinguished in our group of 60 such determinations, and to discuss the bulk of our data by reference to such a chart. Special illustrative curves will be presented after the generalized data. Fig. 1 shows a "mapping out" of convenient areas (outlined by dark lines) into which nearly all individual curves can be allocated.

These areas have been designated tentatively by the terms "saturated," "low normal," "high normal," and "undersaturated," but the distribution of subjects within these groups must vary somewhat according to location and

dietary habits. In view of such experiments as that reported by Crandon and Lund,[23] in which the plasma ascorbic acid was reduced (experimentally) to zero without clinical signs of scurvy or other pathology, it is in fact difficult at the present time to define sufficiency and deficiency with respect to vitamin C. Since in clinical scurvy the plasma ascorbic acid may not reach the zero level,[14] the clinical syndrome probably also depends on the intake of other dietary factors, and it becomes desirable to be able to detect milder degrees of ascorbic acid deficiency.

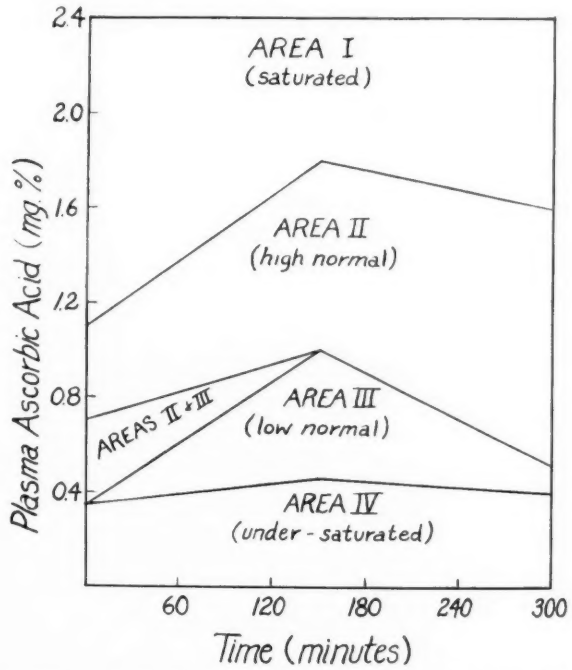


Fig. 1.—Areas chosen to classify types of tolerance curves obtained.

In Fig. 1 it may be noted that there is a small area common to both Areas II and III. This will be discussed later, but it signifies that in spite of a fasting value as low as 0.4 mg. per cent, further characteristics of the curve may, nevertheless, indicate that it should be placed in Area II.

Fig. 2 illustrates some typical curves obtained and their proper allocation in the defined areas which are in this figure represented by dotted lines.

Type I curves, as illustrated, are the type given by "saturated" patients. This type has been encountered only twice among our group of patients, but can be produced at will by feeding extra vitamin C, e.g., 200 to 300 mg. per day for two weeks. In 15 persons possessing among themselves all the other types of curves, we have, by feeding ascorbic acid, converted them into "saturated" persons possessing the type I curve. A typical curve is characterized by a high initial ascorbic acid value, a moderate rise, and generally a failure to decline again in the experimental period. The failure to rise to higher levels is considered to be due to exceeding the kidney threshold (assumed by many[24] to be approximately 1.4 mg. per cent) so that the rate of excretion is equal to or exceeds the rate of intestinal absorption. Thus subjects showing this type of

curve usually excrete in the urine large proportions (60 to 100 mg.) of the test dose. In addition, "saturation" of the tissues prevents these tissues from absorbing any considerable amount of plasma ascorbic acid.

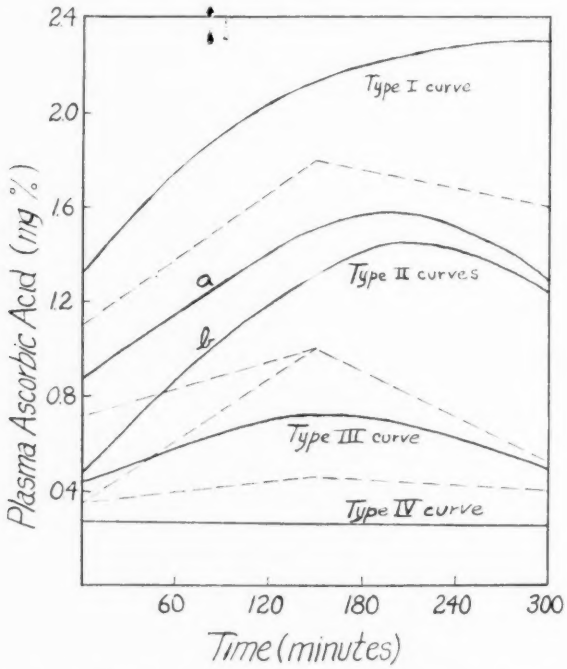


Fig. 2.—Illustrative oral ascorbic acid tolerance curves.

Type II curves, as illustrated in Fig. 2, were found in testing 13 normal nurses (male and female) and 20 hospital patients. All of these had normal dietary habits, being on the regular hospital diet. This group displays many variations in the tolerance curve, but all are characterized by marked rises and various rates of fall. The rise signifies that the rate of intestinal absorption exceeds the rate at which the relatively saturated tissues can absorb ascorbic acid from the plasma, the latter rate displaying itself only after intestinal absorption is more or less complete (latter part of curves). Although urinary excretion (from 15 to 50 mg. during the five hours) parallels the height of the curve in many instances, there were several cases of abnormally higher or lower excretions than that predicted from the curves.

Several persons possessing type II curves could not have been distinguished from patients possessing curves in the next lower state of saturation on the basis of fasting values alone (see type II-b). Thus in our group of 33 type II curves there were 9 subjects whose fasting values fell between 0.35 and 0.7 mg. per cent but who could not be considered from the tolerance curves to be any less "saturated" than those possessing higher fasting values.

Type III curves have been found in four patients of our series and in two other persons during a period of experimental deprivation of vitamin C. The type is characterized by relatively low fasting values (0.35 to 0.7 mg. per cent), with only slight or very transitory rises in the tolerance curve. It is included

as a category, since it is probable that this intermediate type represents a relative unsaturation and will occur quite frequently in the general clinic where patients of more varied dietary habits are encountered.

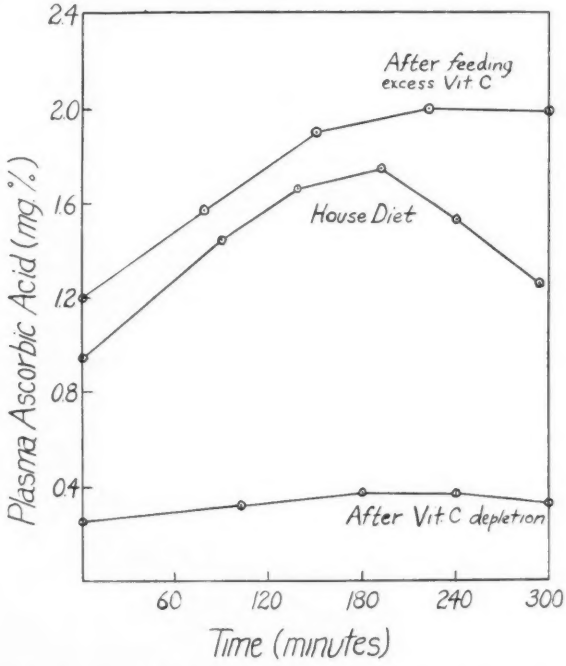


Fig. 3.—Experimental changes produced in tolerance curves of patient N by feeding and deprivation of vitamin C.

Type IV, illustrated in Fig. 2, is typical of vitamin C deficiency. Five of our group, all senile patients, have shown such curves and will be discussed in more detail later. In five experimental persons placed on a vitamin C deficient diet, we have likewise obtained such curves. In such cases the flow of ascorbic acid from the plasma to the unsaturated tissues apparently always exceeds the rate of intestinal absorption. Urinary excretion during the period never exceeds 2 to 3 mg. in these cases. Such curves may not be uncommon in the general population and certainly represent a state of unsaturation requiring vitamin C therapy.

EXPERIMENTAL CHANGES IN THE TOLERANCE CURVE

In order to demonstrate experimentally the variations in the tolerance curve of the same patient on different vitamin C intakes, the tolerance curves of three persons (schizophrenic) were determined on the regular house diet after two weeks of daily administration of 200 mg. vitamin C, followed by seven weeks on a vitamin C deficient diet. Two more patients were placed on the deficient diet. As illustrative of the data obtained, the tolerance curves of one of the first group are shown in Fig. 3, the other data are recorded in Table I. In each case we were able, by feeding ascorbic acid, to produce a tolerance curve, indicating greater saturation, and by deprivation to produce an "unsaturated"

type of curve. Since none of these patients or any others showing type IV curves ever showed any signs of clinical scurvy, the oral tolerance test described here is obviously to distinguish relative degrees of saturation or to differentiate between sufficiency and mild deficiencies.

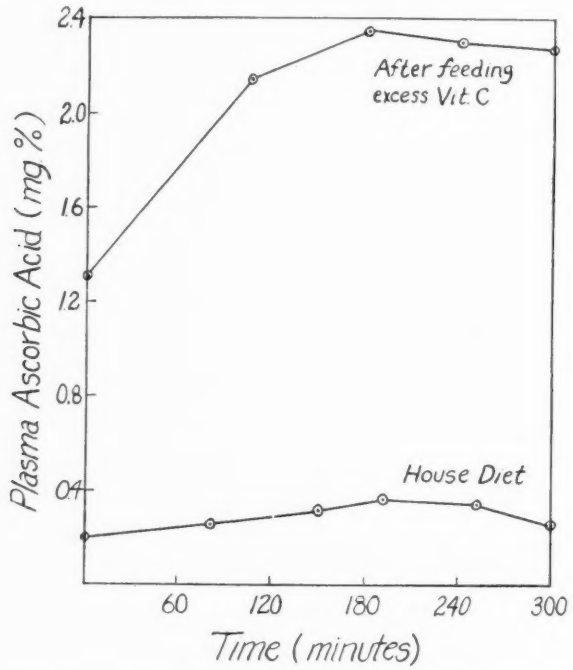


Fig. 4.—Oral tolerance curves on senile patients, before and after supplementary vitamin C feeding.

VITAMIN C AND SENILITY

Other workers[25-27] have given evidence to indicate a vitamin C lack in senility. Although Berkenau[27] has seemed to establish that these findings indicate a true hypovitaminosis C, since such patients required a longer time to "saturate," it was of interest to study such patients with our technique. We have, therefore, carried out the oral ascorbic acid tolerance test on a group of six senile persons. All showed the "unsaturated" or "low normal" (type IV or III) curves. Upon feeding 300 mg. of ascorbic acid per day for two weeks, they showed tolerance curves of type II or I, i.e., curves typical of greater saturation. One example of this is shown in Fig. 4, and the rest of the data are shown in Table II. These patients had been in the hospital on an adequate intake of vitamin C so that the deficiencies indicated by the initial tolerance curves could not be justified on the basis of dietary habits.

No satisfactory explanation (based on experimental proof) of this "endogenous" deficiency of vitamin C in senility has yet been offered.[27, 28] Our data indicate that the fault does not concern intestinal absorption, since such patients display typical saturation curves after feeding of supplementary vitamin C. The basis of this deficiency is under investigation. In the meantime it seems established that better degrees of saturation can be and should

be maintained in senile patients by oral administration of extra ascorbic acid. That this deficiency may be associated with the pathology of senility rather than with "old age" as such is indicated by the finding that 14 other hospital patients from ages 65 to 90 years showed fasting plasma values over 0.7 mg. per cent.

TABLE I

EXPERIMENTAL CHANGES PRODUCED IN TOLERANCE CURVES BY FEEDING AND DEPRIVATION OF ASCORBIC ACID

PATIENT AND DIET	PLASMA ASCORBIC ACID (MG. %)					TYPE CURVE
	0 MIN.	90 MIN.	150 MIN.	240 MIN.	300 MIN.	
K—House	0.75	1.03	1.45	1.01	0.84	II
K—High vitamin C	1.30	2.02	2.05	2.01	1.95	I
K—Low vit. C	0.31	0.38	0.44	0.47	0.42	IV
W—House	0.97	1.52	1.70	1.85	1.74	I
W—High vitamin C	1.08	1.55	1.77	2.05	2.16	I
W—Low vitamin C	0.33	0.34	0.36	0.41	0.36	IV
B—House	0.88	1.44	1.57	1.54	1.37	II
B—Low vitamin C	0.22	0.30	0.36	0.40	0.36	IV
S—House	0.62	1.12	1.24	1.10	0.93	II
S—Low vitamin C	0.30	0.40	0.42	0.39	0.36	IV

TABLE II

ORAL TOLERANCE CURVES ON SENILE PATIENTS BEFORE AND AFTER SUPPLEMENTARY VITAMIN C

PATIENT AND DIET	PLASMA ASCORBIC ACID (MG. %)					TYPE CURVE
	0 MIN.	90 MIN.	150 MIN.	240 MIN.	300 MIN.	
F—House	0.26	0.25	0.29	0.33	0.37	IV
F—High vitamin C	1.11	1.36	1.56	1.76	1.78	II
C—House	0.28	0.29	0.27	0.27	0.28	IV
C—High vitamin C	0.86	1.17	1.46	1.66	1.55	II
W—House	0.24	0.38	0.58	0.54	0.47	III
W—High vitamin C	1.21	1.76	1.83	1.86	1.84	I
R—House	0.34	0.38	0.44	0.48	0.46	IV
R—High vitamin C	0.41	0.68	1.09	1.07	1.00	II
A—House	0.40	0.46	0.47	0.54	0.60	III
A—High vitamin C	0.69	1.12	1.22	1.19	1.15	II

In our group of 20 schizophrenic patients there have been no indications of vitamin C deficiency.

SUMMARY

An oral ascorbic acid tolerance test has been developed to indicate the relative saturation of vitamin C in human beings. On the basis of 60 such determinations, and on experimental changes produced in the curve upon supplementary feeding and deprivation of vitamin C, four categories of relative saturation have been suggested. Since patients showing the lowest "saturation" that can be demonstrated by this test do not show any signs of clinical scurvy, the test is obviously to detect lesser degrees of ascorbic acid deficiency. The main characteristics of the tolerance curves can be adequately distinguished by a fasting blood specimen and two other specimens in the following five hours. The use of this test seems to overcome the unreliability of a single fasting determination of plasma ascorbic acid, and to eliminate the technical difficulties of urine collection involved in previously suggested tests for the degree of vitamin C saturation.

Application of this test has demonstrated a vitamin C deficiency in senile patients which is not related to dietary habits, and has indicated no disturbance of vitamin C saturation in schizophrenia.

We wish to express our thanks to Doctors Kenneth J. Tillotson and W. Franklin Wood of McLean Hospital, and to Professor A. Baird Hastings of the Harvard Medical School, for their suggestions and criticism in this work.

REFERENCES

1. Sherman, H. C., and Smith, S. L.: The Vitamins, ed. 2, New York, 1931 Chemical Catalog Co., Chapter IV.
2. Sigal, A., and King, C. G.: The Influence of Vitamin C Deficiency Upon the Resistance of Guinea Pigs to Diphtheria Toxin, J. Pharmacol. & Exper. Therap. **61**: 1, 1937.
3. Ecker, E. E., Pillemer, L., Wertheimer, D., and Gradis, H.: Ascorbic Acid and Complement Function, J. Immunol. **34**: 19, 1938.
4. Abbasy, M. A., Harris, L. J., Ray, S. N., and Marrack, J. R.: Diagnosis of Vitamin C Submutrition by Urine Analysis: Quantitative Data, Lancet **2**: 1399, 1935.
5. Harris, L. J., Abbasy, M. A., and Yudkin, J.: Vitamins in Human Nutrition: Vitamin C Reserves of Subjects of the Voluntary Hospital Class, Lancet **1**: 1488, 1936.
6. van Eekelen, M., Emmerie, A., and Wolff, L. K.: The Diagnosis of Hypovitaminosis A and C by the Determination of These Vitamins in Blood, Ztschr. f. Vitaminforsch. **6**: 150, 1937.
7. Baumann, T.: The Determination of the Physiological Range of Vitamin C Saturation of the Body, Klin. Wchnschr. **16**: 1246, 1937.
8. Hamel, P.: The Vitamin C Balance in Humans. II. Saturation Experiments for Determination of the Daily Requirement and of the Saturation Deficit, Klin. Wchnschr. **16**: 1105, 1937.
9. Greenberg, L. D., Rinehart, J. F., and Phatak, N. M.: Studies on Reduced Ascorbic Acid Content of the Blood Plasma, Proc. Soc. Exper. Biol. & Med. **35**: 135, 1936.
10. Mindlin, R. L.: The Relation Between Plasma Ascorbic Acid Concentration and Diet in the Newborn Infant, J. Pediat. **13**: 309, 1938.
11. Portnoy, B., and Wilkinson, J. F.: Vitamin C Deficiency in Peptic Ulceration and Hematemesis, Brit. M. J. **1**: 554, 1938.
12. Kellie, A. E., and Zilva, S. S.: The Vitamin C Requirements of Man, Biochem. J. 33: 153, 1939.
13. Neuweiler, W.: The Question of Vitamin C Requirement, Klin. Wchnschr. **18**: 769, 1939.
14. Yu, T.: Blood Vitamin C Content of Chinese in Peiping, Chinese M. J. **56**: 99, 1939.
15. Butler, A. M., and Cushman, M.: Distribution of Ascorbic Acid in the Blood and Its Nutritional Significance, J. Clin. Investigation **19**: 459, 1940.
16. Hawley, E. E., and Stephens, D. J.: Rate of Urinary Excretion of Test Doses of Ascorbic Acid, Proc. Soc. Exper. Biol. & Med. **34**: 854, 1936.
17. Wright, I. S., Lillenfeld, A., and MacLenathen, E.: Determination of Vitamin C Saturation: A Five Hour Test After an Intravenous Test Dose, Arch. Int. Med. 60: 264, 1937.
18. Ralli, E. P., Friedman, G. J., and Kaslow, M.: An Excretory Test for Vitamin C Deficiency and Submutrition, Proc. Soc. Exper. Biol. & Med. **36**: 52, 1937.
19. Finkle, P.: Vitamin C Saturation Levels in Body in Normal Subjects and in Various Pathological Conditions, J. Clin. Investigation **16**: 587, 1937.
20. van Eekelen, M., and Heinemann, M.: Critical Remarks on the Determination of Urinary Excretion of Ascorbic Acid, J. Clin. Investigation **17**: 293, 1938.
21. Stotz, E.: A Clinical Method for the Determination of Ascorbic Acid in Blood Plasma and Urine, J. Lab. & Clin. Med. **26**: 1542, 1941.
22. Mindlin, R. L., and Butler, A. M.: The Determination of Ascorbic Acid in Plasma, A Macromethod and Micromethod, J. Biol. Chem. **122**: 673, 1938.
23. Crandon, J. H., and Lund, C. C.: Vitamin C Deficiency in an Otherwise Normal Adult, New England J. Med. **222**: 748, 1940.
24. Faulkner, J. M., and Taylor, F. H. L.: Observations on the Renal Threshold for Ascorbic Acid in Urine, J. Clin. Investigation **17**: 69, 1938.
25. Plaut, F., and Bulow, M.: Examination of Cerebro-Spinal Fluid in Diagnosis of Vitamin C Deficiency. Delayed Excretion of Ascorbic Acid in Cases With Low Ascorbic Acid Content in the Fluid, Ztschr. f. d. ges. Neurol. u. Psychiat. **154**: 636, 1937.
26. Monavni, J.: Investigations on the Vitamin C Content of the Cerebro-Spinal Fluid in Mental Disease, Ztschr. f. d. ges. Neurol. u. Psychiat. **157**: 636, 1937.
27. Berkenau, P.: Vitamin C in Senile Psychoses. A Preliminary Report, J. Ment. Sc. 86: 675, 1940.
28. Wortis, H., Wortis, S. B., and Marsh, F. I.: Role of Vitamin C in Metabolism of Nerve Tissue, Arch. Neurol. & Psychiat. **39**: 1055, 1938.

THE EFFECT OF YEAST AND MUSCLE ADENYLIC ACID IN MALNOURISHED PERSONS WITH PELLAGRA AND PERIPHERAL NEURITIS*

RICHARD W. VILTER, M.D., WILLIAM B. BEAN, M.D., AND TOM D. SPIES, M.D., CINCINNATI, OHIO

INTRODUCTION

YEAST and crude liver extracts are effective in the treatment of pellagra beriberi, and riboflavin deficiency because they are excellent sources of the vitamins of the B complex, particularly nicotinic acid, thiamin, and riboflavin. Many less well-known compounds are also present in high concentration and, like nicotinic acid, thiamin, and riboflavin, are components of organic catalysts essential for cell respiration. Adenylic acid is one of these compounds. It is the precursor of adenyl pyrophosphate (cophosphorylase) and is a component of a number of polynucleotides, including the nucleic acids, the coenzymes I and II, the prosthetic groups of xanthine oxidase, d-amino acid oxidase, and the yellow enzyme. These considerations suggest a relation of adenylic acid and the vitamins of the B complex, and indicate its great theoretical importance to cellular respiration and nutrition.

In this study 29 selected patients with nutritional deficiency disease were given adenylic acid prepared either from yeast or from muscle.† The patients have been divided into four groups to facilitate description of the therapeutic results.

Group I. Sixteen pellagrins in relapse. This group consisted of 16 persons, all of whom in previous years had responded to nicotinic acid therapy for pellagra. They had continued to subsist on deficient diets, and in the spring of 1940 again had developed clinical evidence of acute pellagra in relapse. Six of this group had multiple ulcers, extending deep in the tongue or buccal mucous membranes. The fiery red borders of the ulcerations were swollen and sharply demarcated from the surrounding tissue. The craters of the ulcers were covered with grayish-white membranes. Each of these 6 patients complained of severe pain around the areas of ulceration and of generalized burning sensations of the entire mouth and tongue. The remaining 10 patients had no ulcerative lesions of the tongue or oral mucous membranes, but each had characteristic pellagrous glossitis and complained of burning sensations of the mouth and tongue; two of these 10 patients also had riboflavin deficiency, as evidenced by severe conjunc-

*From the Department of Internal Medicine, University of Cincinnati; the Department of Preventive Medicine, University of Texas; and the Department of Medicine, University of Alabama.

These studies were aided by grants from Anheuser-Busch, Inc., New York, and E. R. Squibb and Sons.

Received for publication, December 26, 1940.

†Yeast adenylic acid was furnished through the courtesy of Merck and Co., Inc., Rahway, N. J., the Fleischmann Laboratories, New York, N. Y., and Anheuser-Busch, Inc., St. Louis, Mo. Muscle adenylic acid was supplied by the Ernst Bischoff Company, Ivoryton, Conn.

tival and circumcorneal injection, and cheilosis. None of these lesions improved while the patients were restricted to their usual deficient diet for two weeks of preliminary observation. At the end of this period the administration of adenylic acid was begun. Each person was given intravenously 50 mg. of either yeast adenylic acid or muscle adenylic acid, dissolved in sterile physiologic solution of sodium chloride, twice daily for from one to three days. The solution was injected not faster than 2 mg. per minute. The 14 patients receiving yeast adenylic acid and the 2 receiving muscle adenylic acid reported improvement in strength, and then rapid disappearance of all burning sensations of the mucous membranes within six hours. The ulcerations in the mouths of 6 pellagrins healed within two to five days after the first administration of adenylic acid. Those patients who continued to eat an inadequate diet had recurrences of the ulcerations and a diminution of strength within three to five weeks. When relapse occurred, some of these patients requested more of the "same medicine," despite the discomfort that accompanied the injection. The fiery red color of pellagrous glossitis and the cheilosis and conjunctivitis of riboflavin deficiency were not affected by adenylic acid.

Group II. Four persons with prodromal symptoms of pellagra. Four persons, each of whom had been observed with clinical pellagra in previous years, had continued to eat an inadequate diet. When seen in the Nutrition Clinic this year, they complained of weakness, vague pains, irritability, insomnia, and burning sensations of the skin and mucous membranes. Three were given 50 mg. of yeast adenylic acid b.i.d. and one was given 50 mg. b.i.d. of muscle adenylic acid for from one to three days in the manner described.

Within six hours after the first injection these patients reported an increase in strength and vigor, and disappearance of all burning sensations of the skin and mucous membranes. The dramatic disappearance of anxiety, irritability, and nervousness, which follows the intravenous injection of large amounts of thiamin hydrochloride, was not observed after the injection of adenylic acid in the doses used. Accordingly, after this study a single dose of 100 mg. of thiamin hydrochloride was given intravenously and was followed by relief from these nervous symptoms within a few hours.

Group III. Six persons with peripheral neuritis which was refractory to treatment with thiamin hydrochloride and brewers' yeast. Six patients were selected because of severe refractory peripheral neuritis. Five of these persons were addicted to alcohol; the sixth had had recurrent attacks of pellagrous glossitis for ten years and peripheral neuritis for three years because of faulty gastrointestinal absorption. A number of observers had studied these patients clinically, and had treated the peripheral neuritis for from one to three years. The neuritis had failed to respond to intensive treatment with brewers' yeast for two months or more, or to the intravenous administration of from 50 to 100 mg. thiamin hydrochloride daily for a minimum of fourteen days. Before adenylic acid was administered, neurologic examination of each person showed the following similar abnormalities which had been observed before treatment with yeast and thiamin hydrochloride: Each had severe paresthesias of the feet and legs, both spontaneous and in response to pinprick and stroke, diminished perception of

light touch and vibration in the ankles and knees, and diminished appreciation of the position of the toes. Ankle jerks were absent and knee jerks were hypoactive in every case. Each of these patients received, intravenously, 25 mg. of yeast adenylic acid twice a day for ten to thirty days.

Spontaneous paresthesias temporarily disappeared during the administration of adenylic acid. In one case, for example, hyperesthesia, located at the knee before injection, was found near the ankle during the injection, but within fifteen minutes after the termination of the injection the level was found at the knee again. Similar responses were observed in each patient following each injection, but there was also a gradual and steady decrease in all painful sensations in the legs. Within ten to twenty-one days spontaneous pain and demonstrable hyperesthesia disappeared, perception of light touch improved, and the patients were able to walk without pain, whereas before treatment they could not bear the weight of bedclothes on their feet and legs. In 3 cases the knee jerks changed from hypoactive to active during the period of treatment, and the perception of vibration, previously absent in the knees, returned. Ankle jerks, the perception of vibration over the malleoli, and appreciation of the position of the toes, did not change during the period of observation.

Electrocardiographic tracings taken on these 6 persons, which were normal before injection, showed the following temporary changes during the injection: Tachycardia in 3 persons; P-R interval increases of more than 0.02 second in 4; Q-T interval increases of more than 0.02 second in 2; inverted cone-shaped T-waves in all leads in 1; inverted P-waves in Lead 3 in 1; flattened T-waves in all leads in 3. These alterations disappeared within five minutes after the cessation of the injection.

Group IV. Three persons with ulcerations of the tongue whose diets were adequate and who had no evidence of nutritional deficiency. Three persons were selected for comparison with the patients in group I because of recurrent superficial ulcerative lesions of the tongue for which no cause could be found. Their diets were adequate, they presented no evidence of nutritional deficiency disease, and the appearance of the ulcers bore no relationship to the season of the year. Each of these persons was injected intravenously with 50 mg. of yeast adenylic acid twice a day for from three to five days. At the time of injection there was no change in their sense of well-being and later no change in the appearance of the ulcers. They were given 500 mg. of nicotinic acid by mouth for one week. Still there was no healing in the ulceration or improvement in the burning sensations of the tongue in the regions of ulceration.

REACTIONS OBSERVED DURING THE INJECTION OF YEAST AND MUSCLE ADENYLIC ACID

Immediate reactions of varying degrees of severity, such as were described in normal persons,[1] were invariably observed with both the yeast and muscle adenylic acid preparations. Rapid injections of 5 to 10 mg. of either compound produced, within twenty seconds, a flushing of the face, deep inspiration, transient tachycardia, sensation of suffocation, and impending disaster; whereas slow injection of 2.0 mg. per minute produced mild hyperpnea, tachycardia, abdominal cramps, tingling sensations in the mouth, and mild apprehension.

Patients who had burning sensations in the abdomen and about the eyes and mouth stated that these sensations increased temporarily during the period of injection. Transient increase in lacrimation and injection of the conjunctival vessels were noted in several cases, particularly in patients who had conjunctival congestion. In contrast, the intravenous injection of adenine sulfate had no pharmacologic effect and no apparent physiologic effect.

These observations on the immediate reaction and the electrocardiographic alteration during the injection of adenylic acid support the previous observation of Honey, Ritchie, and Thomson,[2] Rothmann,[3] and Ruskin and Katz,[4] on the effect in human beings of intravenous and intramuscular injections of adenosine and ferrous adenylate, substances which act like adenylic acid in all particulars which we have studied.

SUMMARY AND CONCLUSIONS

1. These observations and those previously made suggest that the role of adenylic acid in human nutrition is important and extremely diversified. Within from two to five days following the daily intravenous administration of 50 mg. of adenylic acid from yeast or from muscle, the ulcers in the mouths of 6 malnourished persons disappeared. In contrast, the ulcerations in the mouths of 3 persons who had stomatitis, but no evidence of dietary deficiency, did not improve following the injection of similar amounts of adenylic acid. The administration of either yeast adenylic acid or muscle adenylic acid to persons with pellagrous glossitis or persons with subclinical symptoms of pellagra was followed by rapid improvement in strength and well-being and disappearance of the burning sensations of the mucous membranes.

2. The intravenous administration of yeast adenylic acid to 6 persons with peripheral neuritis who had failed to respond to brewers' yeast or to massive doses of thiamin hydrochloride, intravenously, was followed by striking clinical changes after a period of from ten days to three weeks. Spontaneous pain and demonstrable hyperesthesia disappeared, perception of light touch improved, and the patients were again able to walk without pain.

3. The intravenous administration of either muscle adenylic acid or yeast adenylic acid produced similar immediate reactions. Because of these reactions and the concomitant electrocardiographic changes, we do not recommend adenylic acid for general therapeutic use. Nicotinic acid and thiamin hydrochloride, given in conjunction with a diet rich in protein and calories, continue to be the most efficient therapeutic measures for most malnourished persons with ulcerative stomatitis and peripheral neuritis.

REFERENCES

1. Spies, T. D., Bean, W. B., and Vilter, R. W.: Adenylic Acid in Human Nutrition, Ann. Int. Med. **13**: 1616, 1940.
2. Honey, R. M., Ritchie, W. T., and Thomson, W. A. R.: The Action of Adenosine Upon the Human Heart, Quart. J. Med. **23**: 485, 1930.
3. Rothmann, H.: Der Einfluss der Adenosinphosphorsaure auf die Herztatigkeit, Arch. f. exper. Path. u. Pharmakol. **155**: 129, 1930.
4. Ruskin, S. L., and Katz, E.: The Therapeutic Action of the Nucleotides. The Treatment of the Whole Blood Picture With Ferrous Adenylate, Ann. Int. Med. **9**: 1549, 1936.

LABORATORY METHODS

GENERAL

FLUORESCENT STAINING TECHNIQUE FOR DETECTION OF ACID-FAST BACILLI*

HOWARD E. LIND, M.SC., C.P.H., AND HOWARD J. SHAUGHNESSY, PH.D.
CHICAGO, ILL.

ACID-FAST staining by the Ziehl-Neelsen method is a classic procedure. There are very few laboratories, either private, institutional, municipal, or state, that do not use this method. In view of the difficulties encountered with the Ziehl-Neelsen method, the introduction of a cold stain, followed simply by a decolorizing solution, is appealing in itself. Such is the procedure of the fluorescent technique.

The remarkable simplicity of the technique and the excellent results reported by Richards and Miller of New York prompted us in the Chicago Branch Laboratory of the Illinois State Health Department to apply it to use in a busy routine diagnostic laboratory under normal conditions.

A demonstration by Dr. Edmund Kline at the American Public Health Association Meeting in Detroit in October, 1940, was indeed convincing. It was not until January that we were able to experiment with this technique on a large scale, at which time accessory equipment was made available to us through the courtesy of the Spencer Lens Company.

The application of fluorescence microscopy to acid-fast bacteria and the use of filament rather than arc lamps are due to two German physicians, Hagemann and Keller. The equipment at present makes the method available for use with any monocular microscope and may be used only with materials whose fluorescence will be excited by the available ultraviolet light and the fluorescence that will pass through a yellow filter to the eye.

During the process of investigation of this method with direct and concentrated specimens, the following claims were kept in mind: (1) An increase in positives. (2) Reduction in fatigue in spite of the use of a monocular microscope. (3) Absence of interfering fluorescing substances. (4) Low cost of initial and upkeep of accessory equipment. (5) Rapidity of the procedure. (6) Practicability in small and large laboratories, hospital, and health department. (7) No specific experience necessary. During the course of this paper I shall try to clarify these claims.

*From the Illinois State Health Department, Chicago.
Read at the Laboratory Technicians' Section of the Tri-State Hospital Assembly Meeting, May 9, 1941.
Received for publication, May 12, 1941.

The method and equipment were developed and tested by Dr. Oscar W. Richards, of the Spencer Lens Company Research Department, and were first applied at the Meyer Memorial Hospital in Buffalo by Dr. D. K. Miller. Eleven sputa in their first series of 100 were found positive with this method which were negative with Ziehl-Neelsen. The fluorescence technique on direct smears had greater sensitivity than on concentrations with Ziehl-Neelsen. Acid-fast bacilli found in a spinal fluid and pus were not demonstrated by Ziehl-Neelsen. They also mentioned that one-third less time is required for preparing and reading smears.

Let us take up the necessary accessory equipment and procedure of the technique. (1) A monocular microscope is necessary. The present source of ultraviolet light is limited so that it would be almost impossible to obtain satisfactory light through a double body tube of a binocular microscope. We understand that this limitation is being worked on by Dr. Richards.* The draw tube on a monocular microscope may be substituted for the binocular draw tubes of a Spencer microscope. (2) The microscope should be equipped with an 8 mm. objective and a 20× ocular or 1.8 mm. objective and a 6× ocular. (3) A source of light which has low voltage and high amperage, such as a Universal microscope lamp of 6.5 volts and 2.75 amperes with clear bulb and transformer, is desirable. Universal lamps Nos. 351 and 353 are suitable because the high amperage and concentrated filament furnish sufficient ultraviolet radiation for use with the monocular microscope. Apparently the less intense low voltage lamps and even many of the larger conventional lamps are not satisfactory because either less ultraviolet light is produced or too much of it is absorbed by the lenses. (4) A blue ultraviolet transmitting filter placed in front of the light source is essential. (5) An aluminum-surfaced mirror sets in front of the microscope mirror. This is necessary, since a silver mirror absorbs the ultraviolet radiation instead of reflecting it. (6) Finally a yellow light filter is placed in the ocular of the microscope. This is a complementary filter necessary to see the acid-fast bacteria which fluoresce to appear yellow.

The staining solution is composed of 0.1 Gm. of Auramine O (National Aniline Company), 3 c.c. of liquefied phenol, and 97 c.c. of distilled water. The decolorizing solution is composed of 100 c.c. of 70 per cent alcohol, 0.5 c.c. of concentrated hydrochloric acid, and 0.5 Gm. of sodium chloride. There is no counterstain.

We know that certain microorganisms are called acid-fast bacteria because they retain the basic dye (whether it be fuchsin or auramine) in a phenol or carbolic acid solution after the remaining material on the slide is made void of color with acid alcohol. The acid-fast bacteria retain auramine, a fluorescent dye, after the surrounding material has been destained with acid alcohol. When they are lighted with ultraviolet radiation, this light is absorbed and reradiated as visible yellow light. Consequently, the bacteria appear as bright self-luminous bodies on a dark, almost black, background. This effect is obtained by the blue ultraviolet transmitting filter on or in front of the light source and a yellow filter in the eyepiece. This arrangement becomes black to the eye because the

*Personal communication.

yellow filter absorbs the radiation passed by the blue filter. (Since silver absorbs ultraviolet radiation, an aluminized mirror is substituted.) The yellow light, emitted by the organisms, since auramine is retained, passes through the eye filter and appears yellow.

Smears are made as usual and fixed by heat, as in the Ziehl-Neelsen method. Auramine is applied for two minutes, washed, and decolorized with acid alcohol for two to four minutes, and then redecolorized in a fresh solution of acid alcohol for at least two minutes, again washed and dried; it is now ready for examination. For staining purposes Coplin jars or individual staining may be used. The latter method is to be preferred. Regardless of which method may be used, it is important to use a clean, fresh solution of acid alcohol as a final decolorizer to insure complete decolorization. There is no danger of overdecolorizing, but there is trouble when insufficiently decolorized.

The matter of focusing may cause a little difficulty at first, but if maximum light is obtained before examination is attempted, this difficulty will be minimized. Place the blue ultraviolet filter on the lamp in front of the light source and focus it onto the center of the mirror. Remove ocular and focus objective as near to the stage as possible; then by adjusting the mirror so that the maximum light shines through the body tube, the slide may easily be focused.

The procedure recommended by Dr. Richards follows that an objective of 8 mm. and a 20× ocular giving a magnification of 400 be used, thus allowing an area 20 times that of oil-immersion lens objective with 10× ocular used ordinarily with the Ziehl-Neelsen method.

The acid-fast bacilli appear as small bright yellow bacilli against a dark background. In a pure culture of tuberculosis absolutely nothing but the organisms appear, the background being nearly black. With smears, direct and concentrated, the degree of various other substances present in sputum fluorescing varies, depending upon the method used in concentrating and any other phase used in the process of preparing the sputum for examination.

EXPERIMENTAL

The method and the equipment recommended have been tested under conditions of a public health department laboratory in which nearly all specimens of sputum for the examination of tubercle bacilli are mailed to the laboratory. A few specimens submitted for pneumonia typing were included in this series as direct smears. The test run included 1,123 concentrated specimens and 98 direct smears with the fluorescence method and with the Ziehl-Neelsen technique. Sputum submitted to our laboratory arrives in cresol; it is autoclaved and then centrifuged. Smears made from the sediment are stained and examined for tubercle bacilli. In addition to the recommended use of the low objective and the high ocular lens various combinations, including the oil-immersion and low ocular, were tried.

RESULTS

As seen in Table I a total of 1,221 specimens of sputa were tested. Of these only 98 were examined by direct smear. Ninety-six were negative and two were positive by each method. The bulk of the specimens examined under con-

ditions of concentration with cresol, autoclaving, and centrifuging did not reveal such striking differences as did the comparisons reported by Doctors Richards and Miller. Of the 1,123 specimens examined above, 998 were negative and 106 were positive by both methods. However, there were twelve specimens positive by the fluorescence method which were negative by the Ziehl-Neelsen technique (a re-examination of one of these specimens by the Ziehl-Neelsen was found to be positive). Very interesting to us, but slightly disturbing, was the fact that in seven sputa positive results were obtained by the Ziehl-Neelsen technique but could not be demonstrated by fluorescence. (Two of these smears were very thin, two were bloody, and one was unsatisfactory. Two, however, were missed completely.)

Not included in the foregoing series were four smears of pus and one spinal fluid which were negative by both techniques.

TABLE I

SPUTUM FOR TUBERCLE BACILLI

NO. OF SPUTA	FLUORESCENCE TECHNIQUE		ZIEHL-NEELSEN TECHNIQUE	
	DIRECT	CONCENTRATED*	DIRECT	CONCENTRATED
96	Negative		Negative	
2	Positive		Positive	
998		Negative		Negative
7†		Negative		Positive
106		Positive		Positive
12‡		Positive		Negative
1,221				

*Concentrated with cresol, followed by autoclaving and centrifuging.

†Two smears were very thin, two were very bloody, one smear proved to contain artifacts (acid fast), not tubercle bacilli.

‡One of these was found to be positive by Ziehl-Neelsen technique on re-examination.

NOTE: Not included in the above were 4 smears of pus and spinal fluid which were negative by both techniques.

DISCUSSION

According to Richards and Miller, "A bright yellow tuberculosis organism is more easily seen against a dark background than the usual red stained bacterium on a blue background." As an individual, I would be inclined to agree, but I do not believe that would be the consensus of opinion of the group here. Unless there has been some experience with the dark-field microscope or some other fluorescence technique, the transformation is not as rapid and as easy as it sounds. One problem encountered is that of a mental approach. One must change his or her concept of tubercle bacilli as they should appear. The recommended use of an 8 mm. objective and 20× ocular lens gives one a wider vision and an area twenty times as large but reduces the magnification of the organisms to less than one-half. This difficulty can be overcome with practice. We would recommend that the beginner in fluorescence technique use the oil-immersion objective and a low ocular 6× or 10× just as for other stained preparations of bacteria. This would permit the eye to become accustomed to change in color contrast, without making it necessary to adjust to changes in changing the shape, size, and form of the organisms. It is true that cedar oil will fluoresce so that the background is tan rather than almost black. Also the point of focusing area or working distance is very narrow. Using the low objective

and high ocular (8 mm. and 20×, a magnification of 400 times), one can concentrate attention on change in size and shape.

There may be claims of fatigue at first because of the dark-field and monocular scope, but if the slide is protected from the glare of surrounding light, this objection is eliminated.

The rapidity with which one can examine these slides is very striking. One-fourth to one-third the time is required with this method compared with the Ziehl-Neelsen method once the smears are made. It is true that each field covers twenty times the area of that under oil immersion and increases the speed of examination, although it is necessary to guard against the tendency to lessen the importance of adequate examination of specimen because of increased area covered.

To overcome the problem of occasional thin slides that reveal nothing and render the focusing of the field difficult, apply a spot of auramine stain on the corner of slides.

Associated fluorescence has been a decided problem and inconvenience during this experiment. The fact that cresol fluoresces and nearly all our sputa arrived in cresol reduced distinct contrast, and nearly all material in the smear fluoresced to a greater or lesser degree. This was partially overcome by increasing the time of decolorizing, using low magnification to find suspicious areas and confirming with the oil-immersion lens and 6× ocular. The fact that cedar oil fluoresces as a dull haze blotted out the definite outlines of associated interfering fluorescing substances, while the tubercle bacilli remained clear, bright, and definite.

There has been some work by Dr. Kline* to the effect that heating reduces the sensitivity of the fluorescence stain. This may help to explain why this series involving autoclaving did not reveal such a striking increase in the number of positives. An increase of even 1 per cent is important.

If only one microscope equipped for the fluorescent technique were available in a laboratory, only one person could look at the slides. On the other hand, several people in the laboratory could look at slides stained by Ziehl-Neelsen technique. It would appear that no time would be saved by the fluorescent method. However, this is only partially true, for by the fluorescence method one individual could look at the same number of slides that two, three, and even four, could observe by the Ziehl-Neelsen method. The accessories to this method do not render the monocular microscope unsatisfactory for routine bacteriologic examination of other smears and cultures. It becomes satisfactory for any staining use by simply changing to another ocular minus the yellow filter.

The accessories are reasonable in price. The blue ultraviolet filter, aluminized mirror, and yellow filter are available for $10.00 from the Spencer Lens Company. A Universal light of low voltage and high amperage costs $23.00.

Other chemicals that will fluoresce very little, or not at all, used in concentrating sputum and yet satisfactory for mailing purposes were investigated.

This investigation revealed that the fluorescent technique for acid-fast bacilli, where applied to sputum concentrated with cresol, was not more satis-

*Unpublished work.

factory than our modification of the Ziehl-Neelsen method. It appears that cresol fluoresces to the extent of interfering with the clear fluorescence of the acid-fast bacilli.

This disadvantage does not apply to direct smears. Our series of direct smear examinations was too small to comment upon intelligently.

SUMMARY AND CONCLUSIONS

A total of 1,221 specimens were examined for tubercle bacilli with the fluorescent and the Ziehl-Neelsen techniques. Of these, 1,123 were specimens concentrated by cresol, autoclaving, and centrifuging. The fluorescence technique for the examination of tubercle bacilli was more sensitive than the Ziehl-Neelsen staining method, increasing positives by 1 per cent, although the use of cresol in concentration produced fluorescence which interfered with the examination of the specimens. Because of the larger areas covered and the ease of examination, the time required to examine the smears was reduced by one-fourth to one-third.

The disadvantages of a monocular microscope, the high degree of miscellaneous fluorescence, and the possible fatigue were counteracted by the advantages of larger microscopic fields, simplicity of technique, rapidity of procedure, and increase in number of positives. The simplicity of the technique permits a larger number of specimens to be handled in a shorter period of time.

REFERENCES

1. Hagemann, P. K. H.: Fluoreszenzfarbung von Tuberkelbakterien mit Auramin, München. med. Wchnschr. **85**: 1066, 1938.
2. Keller, C. J.: Vereinfacter Nachweis von Tuberkelbazillen von Fluoreszenlicht, München. med. Wchnschr. **52**: 2024, 1938.
3. Kline, Edmund K., and Leach, Raymond E.: Fluorescence Technique for Demonstrating Acid-Fast Organisms as Used in the Cattaraugus County Laboratory. Mimeographed sheet of instructions distributed by Dr. Kline.
4. Personal communications.
5. Richards, Oscar W., and Miller, David K.: An Efficient Method for the Identification of *M. tuberculosis* With a Simple Fluorescence Microscope, Am. J. Clin. Path., Tech. Suppl. **5**: 1, 1941.

A QUICK, EASY METHOD OF PREPARING TISSUES FOR MICROSCOPIC EXAMINATION BY COMBINING THE TECHNIQUES OF NELSON AND TERRY*

WILLIAM M. WILSON, M.D., PORTLAND, ORE.

THE purpose of this paper is to report a quick, easy, and inexpensive method of fixing, embedding (blocking), cutting, and staining tissues for microscopic examination. The technique is simple and can be mastered by any office or laboratory assistant in a few hours. With the equipment at hand and a little experience the actual working time necessary for carrying out all technical procedures is less than ten minutes. The only time-consuming part of the method is that required for adequate hardening of the tissue block, but this demands no attention. Hardening sufficient for cutting sections occurs in from two to eight hours, depending on the temperature of the media. Cutting, staining, and mounting for immediate microscopic examination can be accomplished within a few minutes.

I claim no credit for the development of any item in this technique. In fact, the procedure described herein merely represents the manner in which I have conveniently utilized, with minor modifications, the following well-developed techniques—(1) The use of protein for embedding small tissues, developed by I. A. Nelson[1] in 1934. (2) A new principle in the microscopic examination of fresh unfixed tissues, developed by Benjamin T. Terry[2] and first reported in 1926. Terry[3, 4] has since reported numerous improvements in technique.

EQUIPMENT

1. Egg albumen crystals, 4 ounces. Albumin crystals make much better blocks than does powdered egg albumen. Domestic egg albumen crystals are superior to imported preparations of the same. These can be purchased at any baker's or candy maker's supply house.†

2. A sheet of ordinary wrapping cellophane (not waterproof) or patapar parchment paper‡ for making embedding boxes. Either of these can be purchased at most department stores. Patapar is easier to handle and makes a more stable box.

3. A small dish, about the size of a one-ounce ointment jar, is satisfactory.

4. Terry's safety razor blade microtome. Directions for making this inexpensive, clever little apparatus have been published by Terry.[4] In a forth-

*From the Department of Gynecological Pathology, University of Oregon Medical School. Received for publication, May 13, 1941.

†I obtained this material from Gray and Company, 1305 N. W. Davis Street, Portland, Ore.

‡Manufactured by the Patterson Parchment Paper Company, Bristol, Pa.

coming publication Terry will describe his safety razor blade "pocket" microtome. A sharp old-fashioned razor may be used for sectioning, but obviously it is less convenient.

5. Formalin, 40 per cent (neutral or slightly alkaline), 4 ounces.

6. A medicine dropper.

7. Fine-pointed thumb forceps.

8. Clean cover slips and a microconcavity slide or a special slide such as described by Terry.[4]

9. A small slice of silk sponge strapped to a glass slide with waterproof adhesive tape.

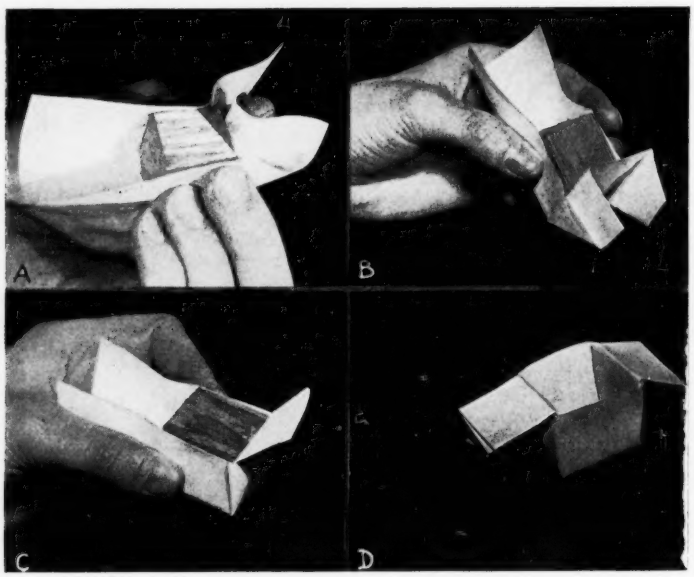


Fig. 1.—An easy method of constructing cubical boxes for embedding purposes. The wood block shown above measures $\frac{3}{4}$ inch in all diameters.

10. A fine-pointed camel's-hair brush or a small inexpensive atomizer for applying stain.

11. Neutralized polychrome methylene blue (Terry)* 0.5 Gm. Place this into a clean 2 ounce bottle and add 50 c.c. of distilled water.

12. A microscope with a good microscopic light. If reflected light is preferred, a 100 watt, frosted, Mazda electric light bulb, provided with a shade or a small photo flood bulb, is recommended.

TECHNIQUE

Fixing and Embedding.—Three grams of egg albumen crystals are dissolved in 10 c.c. of tap water. This quantity of emulsion is sufficient for one large-sized block, or two small ones. Complete solution of the crystals takes from forty-five to sixty minutes, unless the mixture is stirred frequently. This time-consuming factor can be obviated by making up reserve quantities of the emulsion which can be preserved at refrigeration temperatures for several weeks.

*This can be obtained from Benjamin T. Terry, 1001 N. Yakima Ave., Tacoma, Wash.

Containers for blocking are easily made by folding 2½ by 4 inch pieces of cellophane or patapar into the shape of cubical boxes (Fig. 1). The box is placed in a small dish, such as the one-ounce ointment jar. The emulsion of egg protein is poured into the box, and the tissue (fresh or fixed) is placed in the emulsion. A single piece of tissue, as large as one square centimeter or numerous small fragments of tissue, such as small bits of endometrium, can be embedded into a single block. The coagulant, 40 per cent formalin, is poured into the dish around the box. The level of the formalin should be slightly below the level of the emulsoid. By dialysis the formalin gradually permeates the emulsoid, fixing the tissue and coagulating the egg protein to form a solid block. Hardening sufficient for cutting requires from two to eight hours, depending on the temperature of the media. (After a little experience in palpating blocks, the degree of hardness sufficient for cutting is easily determined.)

Fig. 2.—Low-power view of an endometrial polyp.

In an incubator at 140° F. a sufficient hardening can be secured in about two hours, while at a temperature of 70° F. adequate hardening may take from eight to ten hours. The box containing the egg emulsion and tissue may, however, be left in the formalin for twenty-four hours or more without danger of excessive hardening. When sufficiently hardened, the block is held in running water for a few seconds to remove the excess formalin. The cellophane or patapar wrapper peels off easily. After trimming, the block is ready for cutting. When small fragments of tissue, such as strips of endometrium, are embedded, it is usually more convenient to divide the block into two or more squares before sectioning. If it is not convenient to cut sections immediately, the block should be immersed in water to prevent drying and cracking. Any portion of a block before or after cutting can be preserved in tap water for days, or in 5 per cent formalin for months. Tissue embedded by this method is easily re-

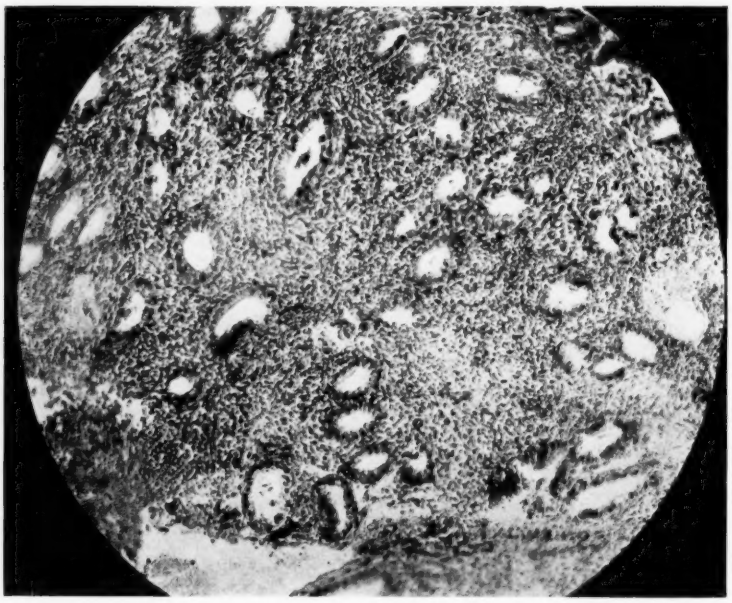


Fig. 3.—Medium-power view of endometrium secured by suction curettage. Chronic endometritis.

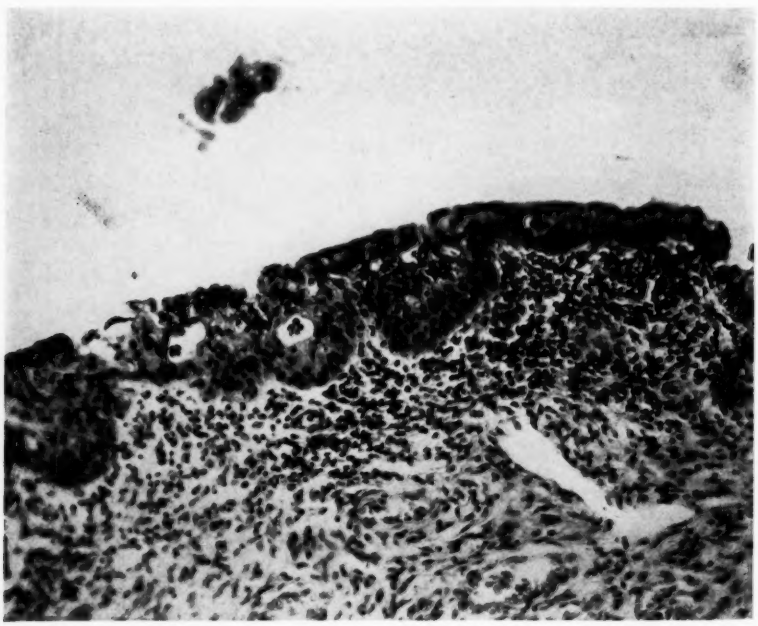


Fig. 4.—High-power view of cervical tissue (biopsy). Chronic cervicitis.

moved from the embedding substance and can be reblocked (for the purpose of securing permanent sections) by some other means, such as the slow paraffin method of impregnation.

Cutting.—The block can be sectioned easily by freehand cutting with an ordinary, sharp old-fashioned razor. With little experience satisfactory sections, measuring a fraction of a millimeter in thickness, can be obtained. Uniformly thinner and better sections can be obtained, however, by employing Terry's[4] safety razor blade microtome. Wetting the block frequently facilitates cutting. Sections may be washed and stained immediately after cutting or they may be placed in water to prevent drying until convenient to stain.

Staining.—Terry's neutralized polychrome methylene blue is a most satisfactory preparation. Only one surface of a section is stained. Overstaining or staining more than one surface hinders proper transillumination and clear visualization of the tissue. To stain, place a section on a thin slab of wet sponge. If not previously washed, gently spurt a few drops of water on the exposed surface. Excessive water will be absorbed by the sponge. The stain may be painted on with a fine-pointed camel's-hair brush or sprayed on with an ordinary atomizer. Adequate staining is secured in from one to three seconds. The intensity and depth of staining are controlled by washing with water. As a rule, properly stained sections are secured by washing almost immediately after applying the stain. Washing is easily accomplished by spurting a medicine dropper or two full of water upon the stain-covered surface. The underlying sponge, if sufficiently wet, will absorb excessive stain and prevent staining of the undersurface of the section. After staining, the section must be mounted and examined immediately, since it fades fairly rapidly.

Mounting.—The section is placed on a cover slip with its stained surface next to the glass. The cover slip is then inverted and mounted on an ordinary microconcavity slide with the section in the cavity. It is now ready for transillumination and examination. If difficulty is experienced in transillumination, the section either is too thick or is overstained.

Examination.—While this method of preparing sections is most satisfactory for the examination of tissues under low-power magnification, excellent high-power views of most tissues can be obtained if the sections are thin enough and stained properly. Photomicrographs of sections prepared by this technique are generally unsatisfactory because of thickness of sections and the tendency of the stain to fade rapidly. Figs. 2, 3, and 4 are photomicrographs of sections photographed immediately after staining. Obviously, the interpretation of sections prepared by this method requires as much knowledge of histology and pathology as is needed for the correct interpretation of sections prepared by other methods.

SUMMARY

1. A technically simple, inexpensive method of preparing tissues for microscopic examination is presented.

2. The method is applicable in the office as well as in the laboratory.

3. Because of the time required for embedding, the method cannot be compared with frozen section technique for speed.

4. With little experience sections can be secured that are as good as, if not better than, the average frozen section.

5. Serial sections of biopsy specimens can be secured quickly and at less expense than by any other method I know.

6. This method should be particularly useful in the office practice of surgeons and gynecologists, who through adequate training, have acquired a good knowledge of micropathology. For instance, in my office it has been particularly useful for the examination of biopsy specimens of lesions of the vulva, vagina, and cervix, as well as for the examination of endometrium obtained by suction curettage.

REFERENCES

1. Nelson, I. A.: The Use of Proteins for Embedding Small Tissues for Sectioning by the Frozen Method, J. Lab. & Clin. Med. **20**: 964, 968. 1935.
2. Terry, Benjamin T.: A New Principle in the Microscopic Examination of Fresh Unfixed Tissue, Proc. Staff Meet., Mayo Clin. **1**: 37, 209, 1926.
3. Terry, Benjamin T.: Improvement in Technic and Results Made on Examining Microscopically by the Razor Section Method 2,000 Malignant Tissues, J. Lab. & Clin. Med. **14**: 519, 1929.
4. Terry, Benjamin T.: An Inexpensive, Easily Made, Safety Razor Blade Microtome With Technic Especially Useful in the Immediate Microscopic Diagnosis of Tissue, Am. J. Clin. Path. **7**: 69, 1937.

I wish to acknowledge my indebtedness to Dr. Benjamin T. Terry for his helpful interest and counsel.

A STUDY OF TYPE HEMOLYTIC REACTIONS OF HUMAN RED BLOOD CELLS IN DISEASE*

Elsie Wieczorowski, Ph.D., and H. R. Fishback, M.D., Ph.D.
Chicago, Ill.

OF THE many methods and great variety of reagents that have been used to test the fragility of red blood cells only one has been found to have practical value as an aid in diagnosis. Hypotonic solutions of sodium chloride are used to test the fragility of erythrocytes in hemolytic jaundice and obstructive jaundice and have been reported as a prognostic guide in pneumonia.[1] This test has been applied to many diseases, but a review of literature does not reveal any systematic study in which several types of hemolytic agents were used to test the strength of red blood cells from a number of different diseases. This study is based upon the use of a comparatively short method which is adaptable for the use of several kinds of hemolytic agents. The method has been applied to red blood cells from normal individuals and patients with various diseases.

REAGENTS

As a neutral salt sodium chloride was used in hypotonic solutions. Its hemolytic property is believed to be due to osmosis, although various explana-

*From the Department of Pathology, Northwestern University Medical School, Chicago.
Received for publication, May 13, 1941.

tions are presented concerning its mode of action.[2, 3] Because it has been so widely used by other investigators, it is a good "yardstick" for comparison of results.

The hemolytic properties of acids and bases are believed to be due to a more complex action. It may be that they change the physical state of the cell proteins[4] or that the action may be concerned with the respective H and OH ions of the acids and bases.[5] Lactic acid and sodium carbonate were chosen to represent the acid and base hemolysins, respectively, because they are less toxic than the stronger agents of this group. A reaction which took place between lactic acid and the hemolysis products clouded the system and made accurate readings difficult. Because of this, it was excluded from this report.

Sodium oleate was used as a representative of the soaps whose lytic properties are now attributed to their action on proteins.[4] This is contrary to the former interpretation, which considered the effects of those active hemolysins to be derived from their lipoid solvent characteristics.

Saponin represented the glucoside lysins. Its hemolytic properties are probably due to its reaction with the cell proteins to a far greater extent than with the lipoids.

Alcohol was used as a lipoid solvent hemolysin. It is a rather weak hemolysin, but gives a very definite end point. A protein precipitate forms in the system a short time after hemolysis is complete, but does not enter into consideration within the time limits of the method to be described.

METHOD

From 3 to 5 c.c. of blood were drawn from the antecubital vein into dry, sterile syringes and transferred immediately to sterile test tubes containing potassium oxalate. It was found that this anticoagulant does not alter the lytic properties of red blood cells. The cells of the fresh, oxalated blood were washed four times in 0.9 per cent sodium chloride solution and centrifuged ten minutes after each washing at a medium speed. At the rate used (1,400 to 1,500 r.p.m.) there is very little mechanical damage to the cells. An average of 2.4 c.c. of packed cells was recovered from 5 c.c. of normal blood. Suspensions of washed cells were prepared in the proportion of 2.4 c.c. of erythrocytes to 97.6 c.c. of 0.9 per cent sodium chloride solution.

TABLE I

AGENT	PER CENT CONCENTRATION									
Test tube No.	1	2	3	4	5	6	7	8	9	10
Sodium chloride	0.075	0.150	0.225	0.300	0.375	0.450	0.525	0.600	0.675	0.750
Saponin (×10-2)	0.833	0.667	0.500	0.433	0.333	0.287	0.250	0.220	0.200	0.180
Sodium carbonate	0.225	0.180	0.135	0.090	0.067	0.037	0.030	0.022	0.015	0.007
Sodium oleate (×10-2)	0.125	0.100	0.083	0.067	0.050	0.042	0.033	0.025	0.017	0.008
Ethyl alcohol	28.50	23.75	20.58	15.83	12.67	7.92	6.33	4.75	3.16	1.58

A series of ten hemolysis tubes was set up for each lysin, consecutively, using varied quantities of stock solutions with 0.9 per cent sodium chloride as diluent. These tubes, together with a tube of red blood cell suspension, were placed in a constant temperature bath set at 37° C. ± 0.2 degree. When the contents of the tubes reached a temperature of 37° C., an equal quantity of

the suspension was added to each tube of lysin and mixed by inverting once. All measurements were made with dry, chemically clean, burettes and pipettes. Care was used to keep all conditions as uniform as possible.

Table I shows the concentrations of each lysin.

The insulated water bath was arranged so that a strip of glass was left free across the front and back, with an equal distribution of light passing through the container. If hemolysis was completed in less than thirty minutes, the time necessary for completion was recorded. It is relatively simple to read complete hemolysis because the contents of the tube become clear. At the end of a half-hour period the degree of hemolysis in all tubes was estimated and recorded. The thirty-minute period proved optimum for securing hemolysis in a fairly wide dilution range of the lysins, and allowed less time for a new colloid-crystalloid adjustment in the cells, or for change of any hemolysis products set free in the tubes.

RESULTS

Average values for each lysin were first made by testing their effect on erythrocytes of normal individuals. Thirty-five persons were used, ranging in age from 5 to 84 years. The level of hemolysis of different individuals varied considerably, but variation in age had no significant effect on the reaction of the cells.

TABLE II

COMPLETE HEMOLYSIS

	NO.	SAPONIN		SODIUM CARBONATE		SODIUM OLEATE		ETHYL ALCOHOL		SODIUM CHLORIDE	
Normal	35	0.342		0.066		0.055		15.24		0.219	
Pernicious anemia	9	0.344		0.074		0.053		15.48		0.175	+
Secondary anemia	8	0.327		0.078		0.062		15.04		0.206	
Obstructive jaundice	4	0.310		0.084		0.064		15.83		0.113	+
Pneumonia	10	0.285	-	0.064		0.052		15.83		0.195	
Scarlet fever	10	0.341		0.072		0.062		14.88		0.165	+
Sepsis	9	0.372		0.073		0.064		16.77		0.175	+
Meningitis	10	0.314		0.055		0.057		14.25		0.188	
Tuberculosis	10	0.373		0.072		0.054		15.83		0.203	
Carcinoma	10	0.383		0.076		0.063	+	15.83		0.188	
Sarcoma	9	0.355		0.082		0.055		16.01		0.175	+
Syphilis	9	0.319		0.071		0.053		15.48		0.225	
THRESHOLD OF HEMOLYSIS											
Normal	35	0.195		0.020		0.036		10.04		0.456	
Pernicious anemia	9	0.193		0.015		0.026		7.91		0.525	
Secondary anemia	8	0.193		0.024		0.033		9.10		0.450	
Obstructive jaundice	4	0.180	-	0.024		0.035		10.29		0.375	+
Pneumonia	10	0.197		0.031		0.035		11.55		0.353	+
Scarlet fever	10	0.194		0.014		0.038		8.23		0.420	
Sepsis	9	0.207		0.017		0.038		8.79		0.456	
Meningitis	10	0.180	-	0.009	-	0.034		9.49		0.435	
Tuberculosis	10	0.180	-	0.012		0.030		10.92		0.398	
Carcinoma	10	0.204		0.023		0.037		8.86		0.450	
Sarcoma	9	0.193		0.024		0.031		9.49		0.458	
Syphilis	9	0.191		0.029		0.038		9.85		0.400	

± indicates statistical significance.

Tests were then made of the red blood cells of patients with various diseases. Table II gives a summary of the average values for beginning and completion of hemolysis.

DISCUSSION

In analyzing Table II two conditions are evident which lower the statistical significance of the results. One is the limited number of cases studied, only 35 for the normal figures, and still less for results in each of the diseases studied. It is realized that increasing the number of cases taken at random improves the statistical accuracy. Practically, however, one is limited as to the number of patients available. Furthermore, the objection as to numbers of cases is mitigated by the rather low swings from average in the individual case studies.

A further statistical objection relates to the lack of fineness of gradation in the hemolysis tests themselves when only ten tubes are used. However, the tests were formulated so that the hemolyzing zone would be found in the central tubes in normal cases. A swing to the right or left in abnormal cases would still be recorded within the given number of tubes. In a very large series of tests, adding still further to the number of tubes obviously would improve the statistical evidence of trends in the results within narrow limits, but would not improve the accuracy of the test.

It appears from the recorded results that the red blood cells are rather well adjusted to possible changes in the blood during disease. At least there is little evidence of variation from normal in the resistance of the cell when exposed to substances which affect either osmotic factors or protein or lipoid constituents of the cells. Even when the averages were altered significantly, the range of concentration at which hemolysis occurred was, in most instances, within the range at which cells from normal persons were hemolyzed.

This seems to be the case in reports of other investigators also; that is, few have found any consistent reaction of the red blood cells that would be of diagnostic value. Trends are indicated but individual cases often vary from increased to decreased resistance when testing several cases with the same disease, as reported by Hill[6] and Simmel[7] on pernicious anemia. Randall[8] found the red blood cell resistance in pernicious anemia to be decreased, while Giffin and Sanford,[9] and Cassells[10] report an increased resistance. On the other hand, Daland and Worthley[11] record normal maximum and decreased minimum resistance. All these investigators used hypotonic solutions of sodium chloride to test fragility. When saponin was the hemolysin, the reaction either was normal or showed a decrease in resistance.[12-14]

Needles[1] found a definite relationship in pneumonia between the resistance of red blood cells to hypotonic sodium chloride and the amount of lung tissue involved. Although there is a significant increase in mean resistance indicated in Table II, the data in these experiments show that many cases fall within normal range, and there is no such correlation of the resistance and extent of lung involvement.

Different results might have been obtained had the cells been unwashed, in which case possible changes in the serum would have played an important part. However, since this group of experiments was on the cells themselves, the serum had to be eliminated by washing in order to make the conditions of the experiment as uniform as possible.

SUMMARY

A method for testing fragility of red blood cells with a variety of type hemolytic agents is presented.

The hemolytic level of human red blood cells with various lysins is indifferent to the factor of age, from 5 to 84 years.

Results of applying this method to red blood cells from patients with pernicious anemia, secondary anemia, obstructive jaundice, pneumonia, scarlet fever, meningitis, sepsis, tuberculosis, carcinoma, sarcoma, and syphilis are recorded.

No significant changes of new diagnostic value were found in the erythrocytes with the method employed.

REFERENCES

1. Needles, R. J.: Erythrocyte Fragility in Pneumonia, Arch. Int. Med. **57**: 174, 1936.
2. Jacobs, M. H., and Parpart, A. K.: Osmotic Properties of the Erythrocytes. III. The Applicability of Osmotic Laws to the Rate of Hemolysis in Hypotonic Solutions of Non-electrolytes, Biol. Bull. **62**: 178, 1932.
3. Ponder, E., and Saslow, G.: The Measurement of Red Blood Cell Volume. III. Alterations of Cell Volume in Solutions of Various Tonicities, J. Physiol. **70**: 169, 1930.
4. Ponder, E.: Erythrocytes and the Action of Simple Hemolysins, Monograph, London, 1924, Oliver & Boyd.
5. Christophers, S. R.: The Mixture Reaction in Hemolysis by Acids and Bases, Indian J. M. Research **17**: 533, 1929.
6. Hill, L. W.: The Resistance of Red Blood Cells to Hypotonic Salt Solutions in Various Anemias, Arch. Int. Med. **16**: 809, 1915.
7. Simmel, H.: Die osmotische Resistanz der Erythrocyten, Deutsches Arch. f. klin. Med. **142**: 252, 1923.
8. Randall, W. L.: Clinical Investigation of Increased Fragility of the Erythrocytes, J. LAB. & CLIN. MED. **23**: 468, 1938.
9. Giffin, H. Z., and Sanford, A. H.: Clinical Observations Concerning the Fragility of Erythrocytes, J. LAB. & CLIN. MED. **4**: 466, 1919.
10. Cassells, D. A. K.: Red Blood Cell Fragility in Various Blood Conditions, J. Path. & Bact. **47**: 603, 1938.
11. Daland, G. A., and Worthley, K.: The Resistance of Red Blood Cells to Hemolysis in Hypotonic Solutions of Sodium Chloride. Observation in Blood Disorders, J. LAB. & CLIN. MED. **20**: 1122, 1935.
12. Bigland, A. D.: The Fragility of Red Blood Corpuscles in Physiology and Pathological States, Quart. J. Med. **7**: 369, 1913-1914.
13. Erlsbacher, O., and Kindermann, F.: Die Saponinresistenz der Erythrocyten bei der Anaemia perniciosa, Ztschr. f. d. ges. exper. Med. **75**: 454, 1931.
14. Ponder, E., and Rhoads, C. P.: Red Cell Resistance in Pernicious Anemia, Proc. Soc. Exper. Biol. & Med. **38**: 540, 1938.

SIMPLIFIED METHOD FOR BIOLOGICAL DIAGNOSIS OF PREGNANCY*

S. Aschheim, M.D., New York, N. Y.

THE biologic diagnosis of pregnancy, which I discovered in 1927, is based on the presence of gonadotropin in the urine and in the serum of pregnant women. My original method is as follows:

For the examination of every urine specimen, one needs five immature female mice, 3 to 4 weeks old and weighing 6 to 8 Gm. The total amounts injected subcutaneously into the five animals vary, but in every case six equal injections are made, distributed over forty-eight hours.

The first animal receives six times 0.2 c.c. (total 1.2 c.c.), the second animal six times 0.25 c.c. (total 1.5 c.c.), the third and fourth animals six times 0.3 c.c. (total 1.8 c.c.), and the fifth animal six times 0.4 c.c. (total 2.4 c.c.). Vaginal smears are made from the third day on. After ninety-six hours the animals are autopsied.

The ovaries are examined with the naked eye, or better, with a hand lens. The presence of hemorrhagic follicles and of corpora lutea is considered indicative of pregnancy. This method gives from 98 to 99 per cent correct results, and only from 1 to 2 per cent erroneous results.

Application of the same method to female immature rats does not give better results than with the mouse. All modifications of the method are based on the same principle, namely, looking for gonadotropin in the urine or the blood.

The modification of Friedman, which uses the adult female rabbit, can give a positive result twenty-four hours after intravenous injection of from 7 to 10 c.c. of urine. The modification of this Friedman method, as used by Brouha, gives the result in forty-eight hours. One injects 5 c.c. of urine on the first day, and 5 c.c. on the second day, into the ear vein of the female rabbit. Ovulation and the presence of hemorrhagic follicles are signs of the action of the gonadotropin.

Other modifications are the ones by Brouha-Simonnet[1, 1a] and Kraus.[2] These authors use immature male mice or rats whose seminal vesicles increase in volume after daily injections of pregnancy urine during seven or eight days. This increase in the volume of the seminal vesicles is caused by the action of gonadotropin on the testicles, especially on the interstitial cells of Leydig, which manufacture the male hormone; this male hormone in turn produces an overgrowth of the seminal vesicles.

Bourg[3] has employed a male rat and a female rat, both immature. He injects 1.1 c.c. of urine daily for five days and studies the seminal vesicles in the male and the ovaries in the female.

*Translated from the French by Alfred Plaut, Beth Israel Hospital, New York.
Received for publication, May 24, 1941.

Shapiro and Zwarenstein[4] have employed the South African clawed toad, *Xenopus laevis*, as a test animal. After injection of pregnancy urine, the animal will lay eggs after ovulation has been stimulated. The result can be studied six to eight hours after the injection. Elkan[5] has obtained satisfactory results with this method.

If I have tried to find a simplication of the method, my reason for this was not the hope of improving the results. External factors have induced me to look for a more economical method as far as material, working time, and expenses are concerned, in short, a method adapted to present conditions. The reasons are the following:

My original method with the five mice, and its modifications, require much work because injections have to be made frequently, and the same applies to the exploratory laparotomy in the female rabbit.

TABLE I

	NO. OF CASES	RAT TEST	RABBIT TEST	REMARKS
Normal pregnancy 6-8 weeks 9-12 weeks 13-20 weeks 28-36 weeks Unknown	 13 11 6 4 1	Of these 35 tests, 33 were positive, none negative. Two rats died	24 positive 2 negative 2 rabbits died In 7 cases no rabbit test was made	Two false negatives with the rabbit test
Abnormal pregnancy 3 months tubal, one day after operation for rupture	1	Positive	Not made	
Two months tubal gestation	1	Positive	Positive	
Same case—5 weeks later	1	Negative	Negative	Operation revealed an old tubal abortion
Missed abortion (intrauterine)	1	Negative	Negative	
Nonpregnant women (amenorrhea, menorrhagia, menopause, castration, myomas)	30	Negative	10 not made 17 negative 3 positive	Three false negatives with the rabbit test In the menopause and after castration the rat test was negative even with 1 c.c. of urine
Teratoma of testicle	1	Negative	Negative	

NOTE: There were no false results with the new rat test (two rats died).

Buying the animals is expensive. That is why I have put to myself the question how it is possible to simplify the method by using immature female rats four to five weeks old.

Applying the original method to immature rats has never appeared advantageous to me. The hemorrhagic follicles are often absent, and sometimes the corpora lutea are difficult to distinguish from the interstitial tissue with the naked eye.

Why then did I choose the female rat as a test animal?

The Third International Conference on the Standardization of Hormones of the Health Organization of the League of Nations,[6] at which I was present, recommended creating an international standard for the gonadotropic sub-

stance obtained from pregnancy urine. The Conference decided that the international unit would be defined as representing the specific gonadotropic activity of 0.1 mg. of a standard prepared by mixing numerous samples. This standard has been examined by numerous workers in order to determine its content of rat units.

It is agreed that a rat unit is the number of international units which produces in 50 per cent of the immature rats, injected subcutaneously during two or three days, vaginal keratinization. Formation of keratinized cells in vaginal smears is known as the Allen-Doisy test. It must be understood, however, that preparations of gonadotropin from pregnancy urine must not contain estrogen. The absence of estrogen can be proved by injecting the gonadotropin into castrated animals. Under these conditions the method for determination of gonadotropin in pregnancy urine can be used for commercial preparations in spite of the fact that the method is based on an indirect effect and not on an ovarian change. In the method I have called the simplified method of the pregnancy reaction, I rely entirely upon vaginal smears (the Allen-Doisy test) without entering into the question whether the reaction is caused by gonadotropin in the urine or by estrogen. As I have shown, the estrogen also is present in considerable quantity in the pregnancy urine. It also is possible that both hormones act simultaneously.

The method is the following: To two female immature rats, 4 to 5 weeks old, weighing 22 to 35 Gm. (the weight figures apply only to our strain), one subcutaneous injection of 0.5 c.c. of the urine to be examined is given. Vaginal smears of both rats are made seventy-two, eighty-four, and ninety-six hours after this single injection.

If the urine comes from a pregnant woman, only epithelial cells without free mucus and without leucocytes in the vaginal smear are found after seventy-two hours (often already after sixty-six hours). After eighty-four to ninety-six hours keratinized cells (seldom mixed with a few epithelial cells) are found. The keratinized cells constitute the criterion of the positive reaction.

The animals need not be killed. They may be used for other experiments, for instance castration.

If an autopsy is performed, easily visible follicles in the ovaries are found. Corpora lutea are seldom seen, and the uterus is found enlarged. Since animals of the same age and weight, which have not been injected, may also show visible follicles, this fact is not of diagnostic value. Only the fact that the vaginal smears contain the keratinized cells after a single injection of 0.5 c.c. of urine is important and can be used for the diagnosis of pregnancy.

The following objections may be raised against this method:

1. The Allen-Doisy test proves only an estrogenic effect. In the castrated animal (rat, mouse) it proves that the fluid injected contains an estrogenic substance.

In the noncastrated immature animal the Allen test indicates that in the injected fluid there is either an estrogenic substance acting directly on the vagina or a gonadotropic follicle-stimulating substance which acts by the intermediation of the ovary on the vagina. Or, finally, a hypophyseotropic substance which

stimulates the anterior lobe of the pituitary and makes it produce and secrete follicle-stimulating hormone. This substance thus would act upon the vagina by way of two intermediary organs, namely, the pituitary and the ovary.

Estrogenic substance is not eliminated in the urine in sufficient quantity, so that 0.5 c.c. in a single subcutaneous injection could lead to a positive Allen-Doisy test except in pregnancy. Normal women excrete less than 1,000 units. The presence of from 300 to 500 mouse units per liter indicates abnormally increased secretion of estrogen.

The amount of estrogen also is increased in women who bear granulosa cell tumors of the ovary. Several hundred mouse units have been found per liter of urine, but so far one has not observed an excretion of 6,000 units per liter, and this is the minimum necessary for giving a positive reaction in the immature rat after a single injection of 0.5 c.c. The abnormal estrogen secretion which sometimes is found in women who have follicular cysts, or fibrocystic ovaries, remains below 6,000 mouse units per liter.

Only in the second half of pregnancy does the elimination of estrogenic substance rise to several thousand mouse units (6,000 to 40,000, and higher) per liter of urine.

Thus, if an immature rat, after receiving an injection of 0.5 c.c. of urine of a woman, gives a positive Allen-Doisy test, one can conclude that it was the urine of a pregnant woman that has produced this reaction.

2. One can object that the follicle-stimulating gonadotropic substance of hypophyseal origin, which also is eliminated with the urine, might falsify results. But this elimination usually does not exceed 30 rat units per liter; it seldom reaches 75 rat units in the normal woman. In women with physiologic or artificial menopause (operation or x-ray) the amount of follicle-stimulating hormone is elevated in the urine. But so far no elevation to 2,000 rat units, which would correspond to 4,000 mouse units, has been found. The highest known figures are 1,000 mouse units. One rat unit of the follicle-stimulating hormone corresponds to two or three mouse units; conversely, in the case of gonadotropin obtained from pregnancy urine (placental origin), one mouse unit corresponds to five rat units.

Occasionally women with primary amenorrhea eliminate gonadotropic follicle-stimulating substance in a large amount, but in my experience not more than 1,000 mouse units. Furthermore, clinically in these women who always have an atrophic uterus, the question of pregnancy does not present itself.

3. Increased elimination of follicle-stimulating hormone (and seldom also of luteinizing hormone) in women with cancer of the cervix or cancer of the vulva has been observed. I have examined about 100 such women, most of whom were in menopause. I have seldom found increased elimination in cancer patients who still menstruated. The figures in these cases did not exceed 1,000 mouse units. I have not yet done quantitative studies of these cases with immature rats, but I do not believe that we could obtain a wrong result with this simplified method by injecting only 0.5 c.c. of urine.

4. The cases of persisting corpus luteum sometimes have given a positive reaction with mice or rabbits. These cases are very rare and one has to wait

a long time before one encounters a case in which the differential diagnosis between extrauterine pregnancy and persistent corpus luteum is of importance. In such cases the simplified method perhaps will be better than the other methods.

5. Cases of chorio-epithelioma and of teratomas of ovaries, and of other organs which give a positive reaction in the mouse and the rabbit, have not yet been examined with the rat method. In tumors which contain chorionic cells no biologic method can give a differential diagnosis between pregnancy and tumor.

6. The combined excretion of estrogen and gonadotropin in quantities, which would give a positive reaction, in our present stage of knowledge cannot be expected, except for chorio-epithelioma or teratoma.

The physician must know that such rare cases exist and must consider them during his clinical examination.

The biologic pregnancy test must not replace exact clinical examination. Close collaboration between the physician and the laboratory man is always necessary.

Thus, we have seen that all the objections against the simplified method can be refuted.

I have chosen 0.5 c.c. for injection. In some cases I have injected only 0.2 c.c., and I have obtained positive results, except in one case in which 0.5 c.c. gave a positive result while with 0.2 c.c. the result was negative. I must continue these tests for if a lower dose than 0.5 c.c. should give the same results, all objections against the method would become even weaker. Meanwhile, I propose the dosage of 0.5 c.c.

In five cases of pregnancy I have given a single injection of 1 or 2 c.c. of urine to male immature rats in order to see whether or not the simplified method is also applicable to the male. This method would have to be based on the increase in size of the seminal vesicles (Brouha-Simonnet, Kraus). Since I could not see such an increase ninety-six hours after the injection, I believe that the simplified method cannot be applied to the male immature rat.

REFERENCES

1. Brouha, L., Hinglais, H., and Simonnet, H.: A propos du diagnostic biologique précoce de la grossesse, Liège méd. **23**: 241, 1930; Paris méd. **1**: 221, 1930; Abstr., Gynéc. et obst. **20**: 672, 1929.
1a. Brouha, A.: A propos du test hormonal de la grossesse, Compt., rend. Soc. de biol. **106**: 6, 1931.
2. Kraus, E. J.: Weitere Erfahrungen mit der Samenblasenreaktion (SBR) zur Erkennung der Schwangerschaft aus dem Harn, München. med. Wchnschr. **79**: 214, 1932.
3. Bourg, R.: Nouveau procédé d'application de la réaction de Zondek et Aschheim pour le diagnostic biologique de la grossesse, Arch. internat. de méd. expér. **6**: 119, 1930.
4. Shapiro, H. A., and Zwarenstein, H.: A Test for the Early Diagnosis of Pregnancy on the South African Clawed Toad (Xenopus laevis), South African M. J. **9**: 202, 1935.
5. Elkan, E.: Xenopus Pregnancy Test, Practitioner **140**: 312, 1938.
6. League of Nations Health Organization Memorandum, J. A. M. A. **113**. 2418, 1939.

AN IMPROVED POLYCHROME METHYLENE BLUE EOSINATE*

LOUIS MICHELSON, BETHESDA, MD.

CONSIDERABLE variation in the effectiveness of staining malaria parasites is found in using different lots of Wright's stain purchased on the open market. Frequently malaria parasites stained by Wright's technique are either understained or are otherwise poorly differentiated. Experiments reported here were undertaken in an attempt to produce a stain with a strong affinity for blood parasites. Several eosinates of methylene blue polychromed with various agents were made and tested. The eosinate of methylene blue polychromed with sodium hydroxide was found to be unstable and of inferior staining quality. However, methylene blue polychromed with sodium hydroxide and then treated with sodium bromide, prior to the addition of the eosin solution, proved effective. Six lots prepared by this procedure have given the desired staining effect, and after more than six months each lot is still stable both in powder and in solution. It is believed, therefore, that this technique provides a reliable method for uniform staining of blood parasites.

The stain is prepared as follows: One gram of methylene blue chloride (certified) is dissolved in 100 c.c. of N/100 sodium hydroxide (carbonate free) in a ground glass-stoppered bottle of about 500 c.c. capacity. Best results are obtained when not more than one-quarter of the bottle is filled by the solution during the polychroming process. The solution is heated in a water bath at 55° C. for two and one-half hours. The bottle stopper is loosened slightly before heating and at half-hour intervals the solution is shaken vigorously for about a minute. At the end of two and one-half hours, 1 Gm. of sodium bromide is added and dissolved in the polychromed methylene blue, and the solution is again heated in the water bath for another two and one-half hours, allowed to cool, and filtered.

To the polychromed methylene blue filtrate is added 60 c.c. of a 1 per cent aqueous solution of eosin "Y" (certified water and alcohol soluble), and mixed well with a glass stirring rod. More eosin is added in 5 c.c. amounts until the solution between the precipitated particles has a reddish tinge. This is best observed by letting a little of the precipitate from the solution run down the inside of the upper portion of the beaker. Generally, about 80 c.c. of the eosin solution give the desired maximum precipitation of the eosinate. The precipitate is allowed to stand for from eighteen to twenty-four hours. The precipitate is then collected on hard filter paper in a Buchner funnel with vacuum until the precipitate is sufficiently dry to crack. The precipitate is allowed to dry completely in the incubator at 37° C., and then ground to a fine powder in a mortar. The powder is stored in a small tightly stoppered glass bottle.

*From the Division of Infectious Diseases, National Institute of Health, Bethesda.
Received for publication, May 24, 1941.

In making the stain 0.1 Gm. of the powder is dissolved in 60 c.c. of absolute methyl alcohol, acetone free. The stain is allowed to stand two to three days, occasionally shaken, until the dye appears to be completely dissolved. The stain solution is filtered and stored in a small tightly stoppered bottle.

STAINING TECHNIQUE

1. Cover a thin blood film with 0.5 c.c. of stain and allow to fix for one minute.

2. Dilute the stain with 2 c.c. of phosphate buffer solution, mix thoroughly, and allow to stain for three minutes. A pH range of 6.4 to 7.0 for the phosphate buffer diluent is satisfactory. When suitable diluent is added, an iridescent scum is formed over the stain. Best staining results are obtained with freshly made diluents, not more than one week old.

3. Wash by flooding with distilled water. Air dry and examine.

This technique is simple and rapid, and has yielded uniform and superior results. It is easy to obtain stained preparations free from precipitated stain.

CHEMICAL

A COMPARISON OF THE BODANSKY AND FISKE-SUBBAROW METHODS FOR THE DETERMINATION OF INORGANIC PHOSPHORUS*

JOSEPH M. LOONEY, M.D., AND CORA G. DYER, M.S., M.T.
WORCESTER, MASS.

DURING the course of our investigations of the physiologic functions in schizophrenic patients, a study was made of the serum phosphatase levels. This necessitated a change in the technique of phosphorus determinations from the Fiske-Subbarow[1] method previously used to that of Bodansky.[2, 3] As the phosphorus values seemed to run lower by the new method, duplicate analyses of 19 sera were made by the two methods. The results of these analyses are given in Table I. The values given under the stannous chloride method have been corrected in accordance with the instructions of Bodansky. Because two cases did not have duplicates by both methods, the statistical analysis was carried out for only 17 samples.

It will be noted that despite these corrections the use of the Bodansky technique gives lower values than that of Fiske and Subbarow. The correlation between the two methods is 0.87, and the values of the Fiske method can be calculated from those obtained by the Bodansky technique after applying his corrections by applying the following regression equation:

$$Y = 0.7784\ X + 0.9511$$

where Y = inorganic phosphorus Fiske, and
X = inorganic phosphorus Bodansky corrected.

The equation for the uncorrected Bodansky value is

$$Y = 0.973\ X' + 0.327,$$

where X' is the uncorrected Bodansky value. The error of estimation by these equations is ± 0.18 mg.

The mean value obtained by the Bodansky method was 3.12 ± 0.1 mg. per cent, while that by the Fiske method was 3.31 ± 0.1. These values were from a group of chronic schizophrenic patients and are lower than those found in the acute stage of the disease.[4]

Since the two series show a high correlation, in order to test for significance of the difference between the means, it is necessary to use the expanded formula for the standard error of the difference:

$$\sqrt{E_1^2 + E_1^2 - 2r\ (E_1E_2)}$$

where E_1 and E_2 are the standard errors of the means, and r is the correlation coefficient between the two sets of observations. In this case the standard error

*From the Memorial Foundation for Neuro-Endocrine Research and the Research Service of the Worcester State Hospital, Worcester.
Received for publication, November 3, 1941.

of the difference is 0.051, and since the difference between the means is 0.19, the ratio between these is 3.73, which is highly significant. The probability that this difference could occur by chance is less than 0.0003.

The duplicate determinations by the Fiske method using 1, 2, 4 aminonaphthosulfonic acid as the reducing agent show much closer agreement than do those using stannous chloride. The variance within samples for the first method was 0.0045, while that for the second method was 0.0138. This difference in variance of three times is highly significant in a sample of 17 duplicates, and indicates that the precision of the Fiske method is much higher than that of the Bodansky method.

TABLE I

	SERUM INORGANIC PHOSPHORUS (mg. per 100 ml.)			
	BODANSKY METHOD		FISKE METHOD	
H. A.	3.05	(3.05)	3.32 3.26	(3.29)
E. B.	3.49 3.55	(3.52)	3.78 3.96	(3.87)
A. B.	3.57 3.72	(3.65)	3.96 3.96	(3.96)
S. C.	3.19 3.34	(3.27)	3.48	(3.48)
W. D.	3.99 4.07	(4.03)	4.06 4.08	(4.07)
A. Du.	2.86 3.00	(2.93)	2.84 2.84	(2.84)
A. O.	3.32 3.28	(3.30)	3.16 3.18	(3.17)
T. G.	3.30 3.36	(3.33)	3.60 3.60	(3.60)
G. H.	3.19 3.26	(3.23)	3.46 3.46	(3.46)
A. Da.	2.90 3.19	(3.05)	3.06 3.06	(3.06)
H. H.	2.81 2.69	(2.75)	2.78 2.78	(2.78)
H. K.	2.90 2.84	(2.87)	2.66 2.66	(2.66)
S. L.	3.67 3.47	(3.57)	3.60 3.56	(3.58)
T. M.	3.00 3.05	(3.03)	3.04 3.04	(3.04)
F. P.	3.17 3.19	(3.18)	3.40 3.28	(3.34)
R. A.	3.00 2.90	(2.95)	3.48 3.60	(3.54)
W. B.	2.54 2.04	(2.29)	2.74 2.76	(2.75)
P. B.	2.67 2.71	(2.69)	3.44 3.14	(3.29)
M. C.	2.75 2.77	(2.76)	3.30 3.30	(3.30)

The reduction by stannous chloride was followed in the photoelectric colorimeter. The maximum reduction occurred almost instantaneously and remained constant for approximately ten minutes. Slight variations in the strength of acid or molybdate produce marked changes in the depth of color. These changes are not so important when the unknown is read against a standard solution but can lead to considerable errors when read in a photoelectric colorimeter and

compared to a standard curve. The method, however, is about six times more sensitive than the Fiske method, so that determinations can be made on smaller amounts of material.

The deviation from Beer's law is due to the reduction of the uncombined molybdic acid, and the precautions outlined by Bodansky must be followed in order to minimize this reduction, as shown in the blank. All alkaline solutions must be stored in paraffined bottles and the N 10 sulfuric acid should be kept in a pyrex glass container to prevent the contamination of the solutions by silicates. This effect of silicates has been noted by Roe and Whitmore.[5]

Berenblum and Chain[6, 7] have noted similar difficulties in the use of stannous chloride as a reducer and have devised a method for overcoming this by extracting the combined phosphomolybdic acid with isobutyl alcohol to remove the excess of molybdic acid. This method, though capable of giving accurate results on dilute solutions, requires several extractions and washings, which are somewhat of a nuisance when many determinations must be carried out.

SUMMARY

Duplicate analyses for inorganic phosphorus were made on sera from 17 schizophrenic patients by both the Fiske-Subbarow and the Bodansky methods. Readings were made on a Lange photoelectric colorimeter. The deviation from Beer's law produced in the Bodansky method, using stannous chloride as the reducing agent, is not completely compensated for by the corrections applied by Bodansky. The variance of the Bodansky method is 0.0138 as compared with 0.0045 for the Fiske-Subbarow method.

By the use of a photoelectric colorimeter and comparison with a standard curve, the error of the Bodansky method due to deviations from Beer's law may be kept at a minimum provided that extreme care is taken in duplicating the reagents used. A known phosphorus standard should be included in each series of unknowns as a check on the reagents. If a photoelectric colorimeter is not available, reasonably accurate estimations can be made by application of one of the formulas given to the values obtained with a Duboscq type colorimeter.

REFERENCES

1. Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**: 375, 1925.
2. Bodansky, A.: Phosphatase Studies. I. Determination of Inorganic Phosphate. Beer's Law and Interfering Substances in the Kuttner-Lichtenstein Method, J. Biol. Chem. **99**: 197, 1932.
3. Bodansky, A.: Phosphatase Studies. Determination of Serum Phosphatase Factors Influencing the Accuracy of the Determination, J. Biol. Chem. **101**: 93, 1933.
4. Looney, J. M., Jellinek, E. M., and Dyer, Cora G.: Physiological Studies in Insulin Treatment of Acute Schizophrenia. V. The Blood Minerals, Endocrinology **25**: 282, 1939.
5. Roe, J. H., and Whitmore, E. R.: Clinico-pathologic Applications of Serum Phosphatase Determinations, With Special Reference to Lesions of the Bones, Am. J. Clin. Path. **8**: 233, 1938.
6. Berenblum, I., and Chain, E.: Studies on the Colorimetric Determination of Phosphate, Biochem. J. **32**: 286, 1938.
7. Berenblum, I., and Chain, E.: An Improved Method for the Colorimetric Determination of Phosphate, Biochem. J. **32**: 295, 1938.

ESTIMATION OF VITAMIN D IN BLOOD SERUM*

Vitamin D in Human Serum During and After Periods of Ingestion of Large Doses of Vitamin D

Josef Warkany, M.D., George M. Guest, M.D., and
Frank J. Grabill, M.S., Cincinnati, Ohio

In the two preceding papers of this series[1, 2] a method for the estimation of the vitamin D content of blood serum was described, and data were presented on the vitamin D content of blood sera from persons taking a normal diet without added vitamin D. In 155 samples of such human blood serum the average concentration of vitamin D was found to be 110 U.S.P. units per 100 c.c.; but values between 66 and 165 units per 100 c.c. were regarded as within normal limits, especially when assays of individual samples of serum were based on a small number of line tests. No significant difference was found between the vitamin D content of sera of normal children at ages beyond infancy and the sera of adults.

The present report deals with similar assays of the concentration of vitamin D in the blood serum of adult patients examined at varying intervals during and after periods in which large daily doses of vitamin D were prescribed. These persons were suffering with mild or moderately severe chronic proliferative arthritis of several years' duration. For the opportunity to study them we are indebted to Dr. Stanley E. Dorst, of the Department of Medicine of the University of Cincinnati. While these patients were treated with vitamin D, it was thought that the assay of the vitamin D content of their blood sera at different times might add information concerning the metabolism of vitamin D in the body. The use of large doses of vitamin D in the treatment of chronic arthritis has been described by numerous authors (see review of this subject by Abrams and Bauer[3]). It is not our purpose to discuss here the merits or demerits of this form of therapy, but merely to describe the changes in concentration of vitamin D which were found in the blood serum of these patients following the ingestion of large doses of vitamin D. Assays of vitamin D in single samples of blood serum from children receiving equally large daily doses of vitamin D for various therapeutic reasons have yielded data essentially similar to those reported here.

For use in these investigations two preparations were supplied by the manufacturers. The Winthrop Chemical Company, New York, furnished a solution

*From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati.

Received for publication, December 14, 1940.

of crystalline vitamin D_2 in oil, 1,000,000 units per gram, and the W. S. Merrell Company, Cincinnati, furnished capsules each containing 50,000 units of viosterol in oil.

METHODS

In biological assays of blood sera, which may vary considerably in vitamin D content, it is useful to perform preliminary tests to ascertain the range in which the vitamin D concentration falls. Another assay is then performed by administering to a series of rats different amounts of serum, varying by smaller increments within the range of dosage established by the preliminary test. If the results of duplicate line tests in this series do not prove uniform, a third assay may be necessary. Since several weeks may elapse sometimes before the final results are obtained, it is important to prevent bacterial contamination of the serum and also to prevent deterioration of its vitamin D content. Both objectives have been achieved by freezing and drying the serum, either the whole sample or the portion not used in the preliminary assay. Such dried serum samples can be stored and then dissolved at any convenient time when needed for further assays. The drying of the serum offers another advantage if the vitamin D content of the serum is low. In such cases the amounts of serum required to initiate healing in rachitic rats may be so large that its administration to the rats may be difficult. Since the dried material can be redissolved easily in one-fifth its original volume of water, the volume of the serum to be administered can be greatly reduced. This smaller volume also makes possible the administration of the serum directly to the rats in doses accurately measured by pipette, a method of administration preferable to mixing a test material with the rachitogenic diet.

METHOD AND PROCEDURE FOR DRYING SERUM

A method of drying of biological materials in the frozen state has been described by Shackell[4] as early as 1909. The desiccation of sera and other biological products, including microorganisms, in the frozen state was accomplished by Elser, Thomas, and Steffen.[5] The method was further developed by Flosdorf and Mudd.[6] For our purpose a simple and inexpensive method was desirable, and we used an apparatus and a procedure which are essentially identical with that described by Cooper and Grabill,[7] the only difference being that vials of serum are substituted for the bacterial cultures. In this method the serum is first frozen in a dry ice bath and then dried in vacuo, the water being absorbed by a suitable chemical desiccant. The availability of the apparatus, its simplicity of construction and operation, and the economy in time, together with the satisfactory results obtained, prompted us to employ it.

The equipment for the drying of the serum consists of a vacuum pump, a Woulf Bottle of 2 liter capacity, serum vials with stoppers* (Fig. 1), a desiccant such as "Drierite," glass connections, and other accessories, such as rubber stoppers and pressure tubing. Our routine procedure is as follows:

Five cubic centimeters of human serum are frozen in 10 c.c. vials in a position illustrated in Fig. 1*A*. In order to insure the proper drying conditions,

*The serum vial stoppers were purchased from the F. J. Stokes Machine Co., Philadelphia, Pa.

the liquid serum should never occupy more than one-half the volume of the container. It is essential to establish a relationship between the volume of serum and the evaporating surface; the depth of the serum at the time of freezing should not exceed 15 mm. By rotation the serum may be shelled in the container while freezing in order to increase the evaporating surface during the desiccation process. The dry ice bath is prepared by adding crushed dry ice to a liquid which does not freeze above –72° C.; alcohol or acetone is suitable. When the bath has become completely cooled, which is indicated by the absence of carbon dioxide bubbles, the vials containing the serum are submerged (Fig. 1*A*) and allowed to remain in the bath for approximately thirty minutes. After freezing,

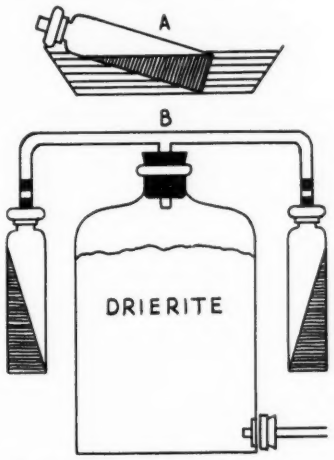


Fig. 1.—Schematic representation of the manner in which blood serum is (*A*) frozen and (*B*) dried.

the serum is quickly connected by means of the rubber stopper to the Woulf Bottle, which has been equipped with tight rubber stoppers and T-tube connections (glass) for the serum vials (Fig. 1*B*). The Woulf Bottle contains approximately 4 pounds of regenerated "Drierite." Simultaneously with the attachment or before, the vacuum pump is started. If the system is free from leaks, the moisture in the frozen serum will sublime to the chemical desiccant. As the moisture evaporates from the surface of the frozen serum, the removal of heat by vaporization is sufficient to retain the serum in the frozen state throughout the period of desiccation. Frost collects on the outside of the containers within seven minutes. The use of the stopcock grease on all connections was found desirable; first, to secure high vacuum throughout the system, and second, to facilitate the rapid attachment of the vials to the Woulf Bottle. The vacuum pump is allowed to maintain the vacuum of the system for a total of eighteen to twenty hours to insure complete drying. At the end of this period the metal sealing bands surrounding the necks of the rubber stoppers are compressed tightly. The rubber connections to the serum bottle are then severed from the T-tubes at a point ¼ inch distal to the compressed band. The serum thus dried and sealed in a vacuum is stored in a cold room until the time of feeding.

Before making use of the process of freezing and drying of blood sera for the purpose of preservation, it was first necessary to determine whether the anti-

rachitic potency of serum was altered by this procedure. A number of sera were assayed immediately after the sample was taken and tested again after various periods of storage in the dried stage. No significant change of the vitamin D content was noticed even after one year of storage.

OBSERVATIONS

Charts 1 to 6 indicate the daily doses of vitamin D administered and the concentration of vitamin D found in the blood serums at different times during and after treatment of each of six patients.

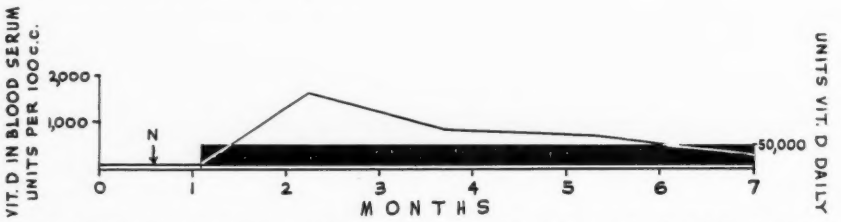


Chart 1.—Vitamin D level in blood serum of W. K. during oral administration of 50,000 units of vitamin D daily. N indicates the average vitamin D level in normal human blood serum (110 units per 100 c.c.)

In all charts the line indicates the vitamin D level in blood serum and the solid black portion indicates dosage.

CASE 1, Chart 1. W. K., white female, 45 years of age, was given 50,000 units of viosterol daily for six months. Due to an insufficient amount of sample, an incomplete assay was made of the blood serum obtained before the treatment was started. It was assumed, however, for making the chart, that the initial concentration of vitamin D in the blood serum was around 110 U.S.P. units per 100 c.c., corresponding to that found in other untreated persons previously reported. In the sample of serum obtained thirty-five days after the start of treatment, the concentration of vitamin D was 1,650 units per 100 c.c. With the same dosage continued, the concentration of vitamin D in the serum progressively decreased to 830, 660, and 330 units per 100 c.c., at two and one-half months, four months, and six months, respectively, after the start of treatment.

CASE 2, Chart 2. A. H. S., white male, 76 years of age, was given 150,000 units of crystalline vitamin D_2 daily during two and one-half months. In one month the concentration of vitamin D in his blood serum rose to 6,600 U.S.P. units per 100 c.c., remained constant eighteen days, and then decreased to 4,400 units per 100 c.c. before the treatment was discontinued. Two and a half months later the concentration of vitamin D in the blood serum had decreased to 1,700 units per 100 c.c. No further blood samples were taken thereafter for assay.

CASE 3, Chart 3. A. M., colored female, 43 years of age, took 180,000 units of vitamin D_2 daily for thirty-one days, and then discontinued treatment for eleven days. She then took 240,000 units of the same preparation daily for twenty-nine days. During the first period of medication the concentration of vitamin D in her blood serum rose to 3,300 units per 100 c.c., but fell to 1,600 units per 100 c.c. at the end of the eleven-day period without medication. At the end of the second period of medication the concentration again was 3,300 units per 100 c.c. Subsequently, the concentration of vitamin D in the blood serum fell to 1,600 units per 100 c.c. at the end of one month, to 800 at the end of two months, and to 330 at three and one-half months after the treatment was stopped.

CASE 4, Chart 4. H. R., colored female, 30 years of age, was given 200,000 units of viosterol daily for five days, then 300,000 units for twelve days, and 400,000 units daily thereafter for four months. The vitamin D in her blood serum increased rapidly to a concentration of 13,000 units per 100 c.c., two and one-half months after the treatment was started. Thereafter the concentration decreased, falling to around 8,000 units per 100 c.c.

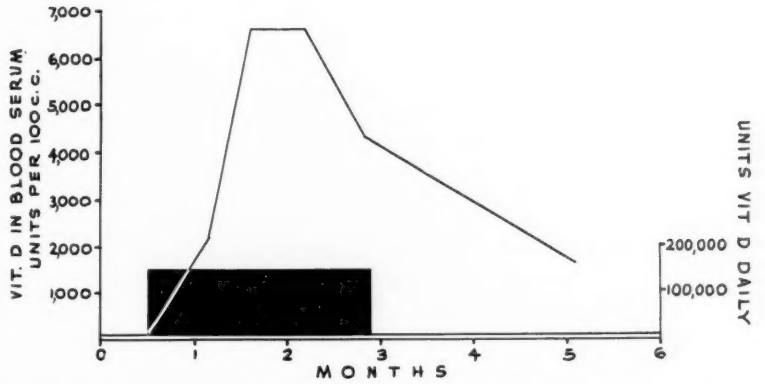


Chart 2.—Vitamin D level in blood serum of A. H. S. after oral administration of 150,000 units of vitamin D daily.

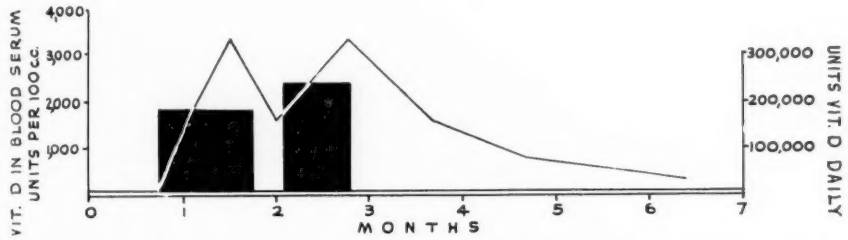


Chart 3.—Vitamin D level in blood serum of A. M. after oral administration of 180,000 to 240,000 units of vitamin D daily.

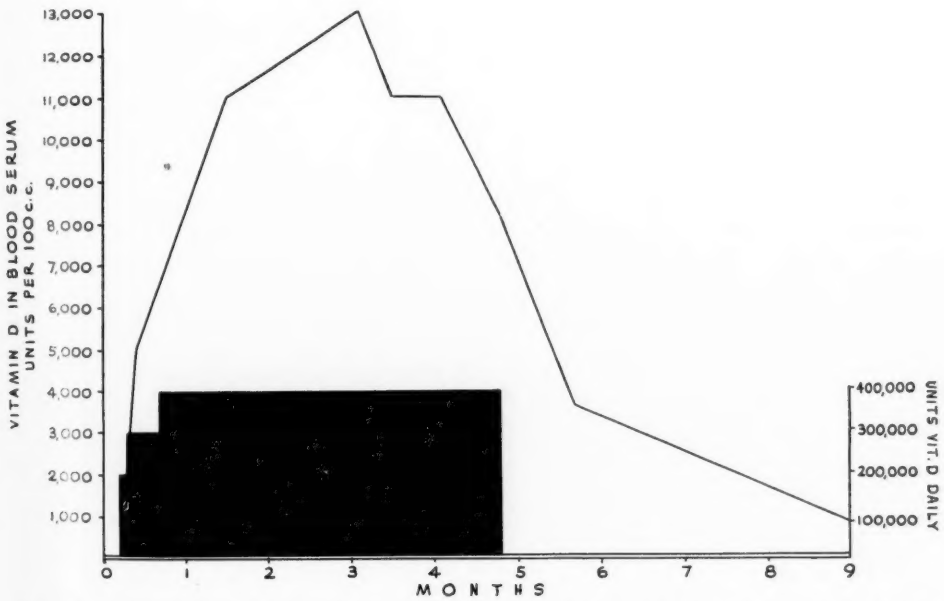


Chart 4.—Vitamin D level in blood serum of H. R. after oral administration of 400,000 units of vitamin D daily.

at the time the treatment was discontinued. After this date the concentration decreased to 5,500 units per 100 c.c. in one month, and to 330 units per 100 c.c. in four months.

CASE 5, Chart 5. E. B., white female, 36 years of age, was given viosterol in doses as follows: 150,000 units daily for three days, 300,000 units daily for seven days, 400,000 units daily for fourteen days, and 500,000 units daily for eighteen days. Thirst and polyuria developed, and the daily dose was decreased to 400,000 units, which she took during the following ten days. She then developed an infection of the respiratory tract and medication was stopped for five weeks. After this interval she again was given 400,000 units of the same preparation daily for forty days; no unfavorable symptoms developed. During the first period of medication the concentration of vitamin D in the blood serum increased to over 9,000 units

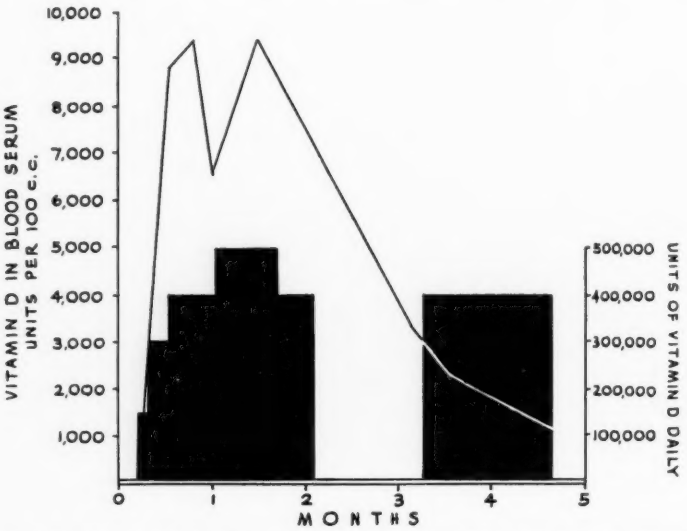


Chart 5.—Vitamin D level in blood serum of E. B. after oral administration of 150,000 to 500,000 units of vitamin D daily.

per 100 c.c., but decreased to 6,600 units per 100 c.c. in the sample taken at the end of the first period when 400,000 units were taken daily. During the period when 500,000 units were taken daily, the concentration again rose to above 9,000 units per 100 c.c. In the sample obtained after thirty days without medication, the concentration of vitamin D in the serum had fallen to 3,300 units per 100 c.c., and this concentration continued to decrease in spite of the resumption of daily doses of 400,000 units. Since this patient lived at home, it is, of course, possible that she did not take the medicine as prescribed, although she claimed that she had taken it faithfully.

CASE 6, Chart 6. G. B., white male, 54 years of age, was given two preparations of vitamin D in three different periods. In the first period viosterol was given daily as follows: 200,000 units for four days; 300,000 units for ten days; 400,000 units for seven days; 500,000 units for fourteen days; and 450,000 units for five days. Because of the development of thirst, polyuria, and slight nausea, medication was stopped. Seventeen days later he was given crystalline vitamin D_2, 450,000 units daily for ten days. Nausea returned and medication was stopped for six months. In a third period viosterol was given in smaller doses: 50,000 units daily for eighteen days; 150,000 units for twenty-five days; and 200,000 units daily for five months. In Chart 6 are represented the changes in concentration of vitamin D in the blood serum during these successive periods with and without medication. In the first period the concentration rose to 13,200 units per 100 c.c., and then fell to 6,600 units per 100 c.c. at the end of the first rest period of seventeen days. During the second period, ten days, the concentration of vitamin D in the serum fell to 4,400 units per 100 c.c., although the patient again developed symptoms of intoxication. During the long rest period the con-

centration of vitamin D in the serum gradually fell to 330 units per 100 c.c. During the third period of medication the concentration of vitamin D in the blood serum rose to 4,400 units per 100 c.c., and then gradually decreased again in spite of the continued constant daily dose of viosterol.

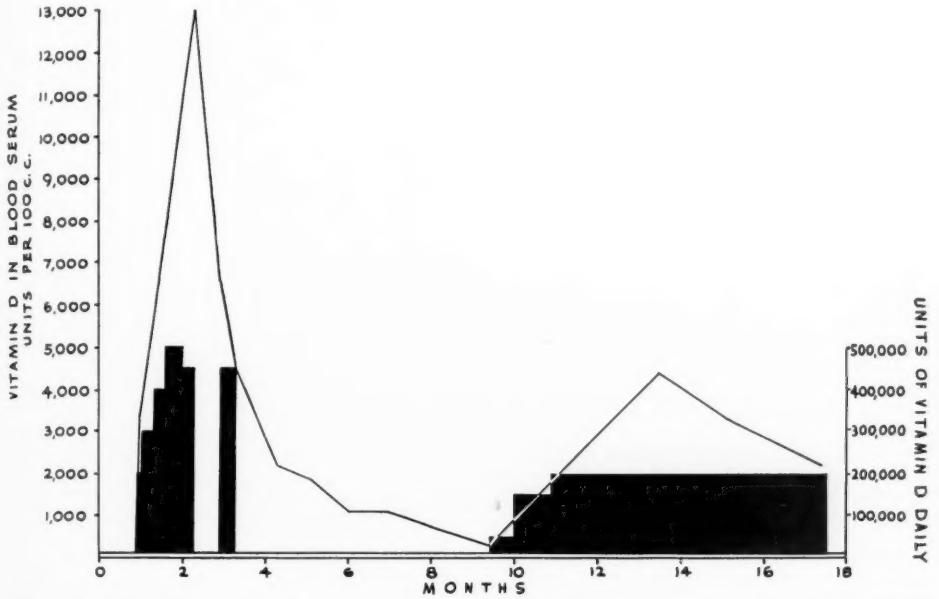


Chart 6.—Vitamin D level in blood serum of G. B. after oral administration of 200,000 to 500,000 units of vitamin D daily.

DISCUSSION

Prolonged oral administration of large doses of vitamin D (50,000 to 500,000 International units daily) to adult patients led to an increase in the antirachitic potency of their blood serum. The highest potency observed was more than one hundred times that of normal human blood serum. Such a concentration of vitamin D in the serum, equal to that of cod-liver oil, was tolerated by patient H. R. for more than two and a half months without any symptoms of intoxication, but another patient (G. B.) complained of thirst, polyuria, and nausea when a similar level of vitamin D in his serum was reached. The concentration of vitamin D in the serum was roughly proportional to the daily dose of vitamin D administered. This fact is demonstrated by Charts 1 to 6, previously discussed, and by data in Table I. Calculations of the vitamin D content of the entire volume of blood serum at the time when the concentration was at its maximum were based on the concentration of vitamin D per 100 c.c. of serum, the body weight, the blood volume (assumed to be $\frac{1}{13}$ the body weight), and the per cent volume of cells in the blood (determined by centrifugation of a sample of heparinized blood, obtained at the same time as the sample used for serum vitamin D determination). In Table I are listed the amounts of vitamin D thus calculated to be present in the entire blood serum of each of six patients, and the daily dose of vitamin D each was taking when the concentration of vitamin D reached the highest level in the serum. In three patients (W. K., H. R., and G. B.) the maximal accumulation of vitamin D determined in the entire blood

serum corresponded closely to the amount that was being ingested daily. Four charts (1, 2, 4, and 6) suggest, however, that the maximal concentration of vitamin D in the serum was not maintained throughout the period during which the administration of large daily doses was continued. In Case 5, when the daily dose of 400,000 I. U. was discontinued, the concentration of vitamin D in the serum promptly decreased, as was to be expected. When the same dose was resumed, the concentration of vitamin D in the serum did not return to its previous level. Such findings might be interpreted to mean a diminishing absorption of the drug, increased excretion, or a more rapid rate of destruction of vitamin D in the body. Further studies would be required to determine which of these explanations is the most probable.

TABLE I

PATIENT	DAILY DOSES (I.U.)	CALCULATED AMOUNT OF VITAMIN D IN ENTIRE BLOOD SERUM OF PATIENT (I.U.)
W. K.	50,000	54,000
A. H. S.	150,000	220,000
A. M.	180,000	110,000
H. R.	400,000	350,000
E. B.	400,000	186,000
G. B.	450,000	460,000

In most of the blood samples drawn from these patients for assay of the vitamin D in the serum, the distribution of inorganic and organic acid-soluble phosphorus in the serum and cells also was determined.* Although these data are not reported, it is germane to note here that the changes in distribution of phosphorus, such as were found in the bloods of these patients during their courses of treatment, were only slight and of doubtful significance. These investigations were made because of earlier findings that in some conditions vitamin D therapy brought about large changes in the concentration of both inorganic and organic phosphates in the blood. In rachitic animals with low concentrations of inorganic phosphorus in the blood serum and of phosphoric esters in the blood cells, the distribution of phosphorus in the blood became normal following the administration of small curative doses of vitamin D[8-10]. In normal rabbits the administration of large doses of irradiated ergosterol led to great increases of inorganic phosphorus in the whole blood and of organic acid-soluble phosphorus in the blood cells,[11] mainly accounted for in the diphosphoglycerate fraction.[12] The doses of irradiated ergosterol, which provoked such changes in the distribution of phosphorus in the blood of rabbits, were, however, much larger in proportion to body weight than the doses administered to the patients here reported. Moreover, the marked chemical changes of the bloods of rabbits were accompanied by definite signs of intoxication, such as loss of weight and hyperazotemia. No attempt was made to increase the doses of vitamin D given the patients to amounts causing more than very mild symptoms of intoxication. It is noteworthy that the concentration of vitamin D in the blood may be increased to many times the normal level without much, if any, change in the distribution of phosphorus in the blood.

*With the cooperation of Dr. S. Rapoport.

SUMMARY

A method of freezing and drying blood serum, which permits preservation and concentration of the serum without alteration of its vitamin D content, is described.

Data are reported on the vitamin D content of the blood serum of six adult patients during and after periods of taking large doses of vitamin D (50,000 to 500,000 International units daily).

During periods in which the daily high dose of vitamin D was constant, the concentration of vitamin D in the blood serum increased rapidly to a maximal level, which was roughly proportional to the daily dose. After doses of 400,000 to 500,000 units daily the concentration of vitamin D in the serum rose to between 9,000 and 13,000 I. U. per 100 c.c.; in other words, to an antirachitic potency equal to that of cod-liver oil. Few toxic symptoms were observed in the patients who took such doses of vitamin D.

During periods in which the daily high dose of vitamin D was constant there appeared to be a tendency for the concentration of vitamin D in the serum to decrease somewhat after attaining a maximal concentration.

After abrupt withdrawal of the high daily doses of vitamin D from three to six months elapsed before the concentration of vitamin D in the blood serum fell to a normal level.

REFERENCES

1. Warkany, J.: Estimation of Vitamin D in Blood Serum, Am. J. Dis. Child. **52**: 831, 1936.
2. Warkany, J., and Mabon, H. E.: Estimation of Vitamin D in Blood Serum. II. Level of Vitamin D in Human Blood Serums, Am. J. Dis. Child. **60**: 606, 1940.
3. Abrams, N. R., and Bauer, W.: Treatment of Rheumatoid Arthritis With Large Doses of Vitamin D. Critical Evaluation, J. A. M. A. **111**: 1632, 1938.
4. Shackell, L. F.: Improved Method of Desiccation, With Some Applications to Biological Problems, Am. J. Physiol. **23**: 325, 1909.
5. Elser, W. J., Thomas, R. A., and Steffen, G. I.: Desiccation of Sera and Other Biological Products (Including Microorganisms) in the Frozen State With the Preservation of the Original Qualities of Products so Treated, J. Immunol. **28**: 433, 1935.
6. Flosdorf, E. W., and Mudd, S.: Improved Procedure and Apparatus for Preservation of Sera, Microorganisms and Other Substances—The Chryochem Process, J. Immunol. **34**: 469, 1938.
7. Cooper, M. L., and Grabill, F. J.: Simplified Apparatus for the Preservation of Bacterial Cultures in the Dried State, J. Lab. & Clin. Med. **25**: 184, 1939.
8. Warkany, J.: Blood Phosphorus During Development and Healing of Rickets; Notes on the Lack of Relationship Between Level of Plasma, Phosphorus and Rachitic State, Am. J. Dis. Child. **49**: 318, 1935.
9. Rapoport, S., and Guest, G. M.: Changes of Organic Acid-Soluble Phosphorus, Diphosphoglycerate, Adenosinetriphosphate, and Inorganic Phosphorus in the Blood Cells of Rats During the Development and Healing of Rickets, J. Biol. Chem. **126**: 749, 1938.
10. Guest, G. M., and Rapoport, S.: Role of Acid-Soluble Phosphorus Compounds in Red Blood Cells in Experimental Rickets, Renal Insufficiency, Pyloric Obstruction, Gastroenteritis, Ammonium Chloride Acidosis and Diabetic Acidosis, Am. J. Dis. Child. **58**: 1072, 1939.
11. Guest, G. M., and Warkany, J.: Effects of Overdosage of Irradiated Ergosterol in Rabbits; Changes in the Distribution of Phosphorus in Blood Cells and Plasma, J. Biol. Chem. **100**: 445, 1933.
12. Guest, G. M., and Rapoport, S.: Effects of Overdosage of Irradiated Ergosterol in Rabbits; Changes of Diphosphoglyceric Acid in the Blood Cells, J. Biol. Chem. **124**: 599, 1938.

DEPARTMENT OF ABSTRACTS AND REVIEWS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

BLOOD: Blood Cells in Healthy Young Infants, Washburn, A. H. Am. J. Dis. Child. 62: 530, 1941.

A description is given of the postnatal readjustments in the red blood cell values of 15 healthy infants during the first ten to eighteen weeks of life.

The simple explanation advanced for the observed changes is based on the comparison of the weekly values for the red blood cell count, the reticulocyte count, and the body weight for each individual baby. The comparison led to the conclusion that the decrease in the red blood cell count, the hemoglobin content, and the volume of packed cells in the first six to nine weeks of life is dependent largely on two factors.

The first factor is the rapid increase in total body mass. This appears to involve a similar rate of increase in total blood volume, so that the ensuing dilution results in fewer red blood cells per cubic millimeter of blood.

The second factor is the abrupt decrease in reticulocytes, indicating a sudden decrease in the rate of production of red blood cells. In most babies the rate of production between the ages of 1 week and 5 or 6 weeks appears to be too low for maintenance of the red blood cell level in the blood, especially in the presence of an increasing plasma volume.

The decrease in the number of red blood cells and in the amount of hemoglobin per cubic millimeter of blood results in a rise in the reticulocyte percentage at about the fifth to eighth week. The sharpness and the persistence of the rise in different babies vary, roughly, with the rapidity of weight gain and the speed of decrease in the red blood cell count.

In most babies the red blood cell count starts to increase between the sixth and the tenth week of life and continues to rise until it reaches a level of between 4,500,000 and 5,500,000 at about 16 weeks of age. Meanwhile, the number of reticulocytes decreases to approximately 1.0 per cent.

It seems probable that the period of postnatal adjustment of the red blood cells for most healthy babies extends over the first fifteen to twenty weeks of life.

LEUKEMIA, Erythroblastic, Stransky, E., and Quintos, F. N. Am. J. Dis. Child. 62: 577, 1941.

The case of a girl, aged 2 years 3 months with chronic myelogenous leucemia is presented. The patient died thirty-one hours after admission to the hospital. The observations on the blood and the bone marrow, as well as the histologic sections, were characteristic of chronic myelogenous leucemia. The most striking feature in the case was the marked erythroblastosis. There were 61,000 nucleated red blood cells per cubic millimeter in the peripheral blood and more than 280,000 in the bone marrow. The majority of the nucleated red blood cells were normoblasts. The clinical diagnosis was chronic myelogenous leucemia, and the hematologic diagnosis was severe erythroblastic anemia with chronic myelogenous leucemia; the final diagnosis in the case was erythroblastic leucemia. The similarity between the data in this case and those in cases of the so-called erythroleucoblastic type of hydrops foetalis congenitus is discussed. The unknown cause of chronic myelogenous leucemia may stimulate not only the granulocytopoietic tissue but, exceptionally, the erythropoietic tissue of the bone marrow.

BLOOD LOSS, Response of Normal Subjects to, Ebert, R. V., Stead, E. A., and Gibson, J. G. II. Arch. Int. Med. **68**: 578, 1941.

Seven hundred and sixty to 1,220 c.c. of blood (15.5 to 19.7 per cent of the total blood volume as determined by the plasma volume and the hematocrit reading) were removed from 6 normal persons in six to thirteen minutes. In 5 of the 6 there developed collapse, characterized by weakness, nausea, blurred vision, pallor, sweating, and fall in arterial pressure. Before the onset of collapse the heart rate increased from 14 to 30 beats per minute. At the height of collapse the heart rate became slow, ranging between 36 and 40 beats per minute.

The plasma volume began to increase immediately after hemorrhage and continued to increase for the next forty-eight to seventy-two hours. At the end of seventy-two hours it was approximately equal to the plasma volume before hemorrhage plus the volume of red blood cells removed.

During the first two hours after venesection the plasma volume was increased by protein-poor fluid, so that the serum protein concentration decreased. Thereafter, fluid and protein were added to the plasma at the same time, and the serum protein concentration remained unchanged, though the total circulating protein increased.

At the end of seventy-two hours approximately one-fourth of the plasma protein was protein which had been added to the plasma after venesection. Loss of blood acted as a physiologic stimulus for the production of normal protein, and the addition of the new protein did not change the proportions of the albumin and the various globulin fractions.

When physiologic solution of sodium chloride was given intravenously immediately after hemorrhage, it was not retained in the blood stream in sufficient quantity to restore the plasma volume to normal.

After hemorrhage the blood volume was not restored to normal until new plasma protein had been added to the circulation.

Hematocrit readings in these experiments accurately reflected the direction of a change in plasma volume.

NEPHROSIS: Acacia in the Treatment of the Nephrotic Syndrome, Goudsmit, A., Binger, M. W., and Keith, N. M. Arch. Int. Med. **68**: 513, 1941.

Intravenous injection of acacia into 4 patients who had the nephrotic type of edema was followed by marked increases in urinary excretion of water and of chloride (expressed in terms of the sodium salt). This increased excretion of sodium chloride and of water brings about a reversal of conditions which is significant and beneficial, considering the excretion by untreated patients. Neither increases in the volume of the circulating blood nor changes of colloid osmotic pressure appear to account for this change in renal function. Experiments on animals have also demonstrated this increased excretion of chloride after intravenous administration of acacia. It is suggested that an analogous effect might well be responsible for the diuretic response of these patients.

FLUIDS, BODY, Suggested Modification of Mandelbaum's Method of Examining, for Cells, Tannhauser, S. Arch. Path. **32**: 450, 1941.

The fluid (pleural or ascitic) is collected with addition of an appropriate amount of an anticoagulant (sodium citrate or potassium or sodium oxalate). After centrifuging the mixture in a large tube with a flat round bottom, a suitable amount of oxalated or citrated plasma is added; double the quantity of the remaining centrifugate has been found appropriate. If, for instance, an oxalated plasma, obtained by adding 0.4 c.c. of 4 per cent potassium oxalate to each 10 c.c. of blood, is used, 10 drops of a 0.5 per cent solution of calcium chloride for each cubic centimeter of plasma will induce clotting. It is important to add foreign plasma and not to rely on the clotting properties of the body fluid, since often this will not clot, owing to previous spontaneous defibrinization. The centrifugate is thoroughly

mixed with the plasma, and calcium chloride is added. In a short time clotting starts and encloses all cells present in the centrifugate. After retraction the clot is transferred to a 10 per cent solution of formaldehyde or Zenker's fluid for fixation, and its further treatment is that of any other specimen, with the fibrin of the clot holding the cells together in a satisfactory way.

When oxalated or citrated plasma is not available, a few centimeters of freshly obtained venous blood may also be used to advantage.

The particles of malignant growth stand out well from the blood clot. In case an examination of the cellular content of the fluid is desired besides examination for cells or particles of malignant growth, that has to be done on a separate smear before addition of the blood.

This modification of the method of Mandelbaum may also be used when examination of sputum for malignant cells is to be made and paraffin embedding of the whole sputum is desired. The procedure then differs somewhat from that described:

In order to prevent disintegration of the cells by the action of the always present bacteria, the sputum is directly collected in a 10 per cent solution of formaldehyde. Usually the mucous sputum balls are dissolved or precipitated in small fragments by the formaldehyde (except when the sputum is highly albuminous; it then is more coherent after fixation with a solution of formaldehyde alone). After fixation of the sputum for twenty-four hours, the particles are centrifuged and washed twice with distilled water; a suitable amount of plasma with calcium chloride solution is then added. The resulting clot is again transferred to a solution of formaldehyde or Zenker's solution for fixation, washed and dehydrated in the graduated alcohols as already described.

FORENSIC MEDICINE: Significance of Dextrose and Non Dextrose Reducing Substances in Postmortem Blood, Hill, E. V. Arch. Path. 32: 452, 1941.

Post-mortem samples of blood for determinations of dextrose should be removed from the left side of the heart if significant errors due to the post-mortem diffusion of dextrose from the liver to the right side of the heart are to be avoided. The dextrose responsible for the post-mortem rise in dextrose on the right side of the heart is liberated by glycogenolysis occurring in the liver. Fasting animals or cadavers presenting marked hepatic damage do not show this post-mortem rise in dextrose.

Glycolysis occurring after death causes a progressive lowering of the dextrose content of the heart's blood. Intravascular and in vitro glycolysis occur at approximately the same rate and are not influenced by clotting of the blood.

The rate of glycolysis was studied in vitro at various temperatures, and the temperature coefficient of the reaction was determined.

The dextrose and nondextrose reducing substances were determined in 73 medicolegal and hospital cases. Significant quantities of residual dextrose were present in the majority of cases of asphyxia, shock, acute coronary closure, rapidly developing anoxemia, increasing intracranial pressure, and fluoride poisoning, indicating that agonal hyperglycemia was present.

For significant results in cases in which hypoglycemia is suspected, specimens of blood for analysis should be taken within two hours after death.

SIMPSON MEMORIAL INSTITUTE
ANN ARBOR, MICH.